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RESEARCHES ON TETANUS[1]

V. Distribution and Fate of Tetanus Toxin in the Body[2]

JOHN J. ABEL, E. A. EVANS, Jr., and BETTYLEE HAMPIL

From the Laboratory for Endocrine Research, the Department of Bacteriology of the School of Hygiene and Public Health, and the Department of Surgery[3] of the Johns Hopkins University

I. INTRODUCTION

From the time, fifty years ago, when it was first conclusively proved that tetanus is due to the action of a highly poisonous substance elaborated by the *clostridium tetani*, at its place of entrance[4] into the

[1] A grant from the Carnegie Corporation of New York has made it possible for the authors to carry on a series of investigations on tetanus.

[2] The present paper, much of the experimental work of which was completed three years ago, as may be seen from the protocols, does not follow the projected order of our papers, as set forth on page 348 of Paper IV. It was there stated that Paper V would furnish further experimental proof of the peripheral muscular action of toxin and that Paper VI would furnish similar proof for its purely central action. This announced order of our forthcoming papers was not held to because it became apparent that the ground would be better cleared for our later papers by publishing at this time the many facts that have been brought to light by our assays of the toxin content of the blood. It will be apparent that no one aspect of the singular disease, tetanus, can be viewed by itself as an entity, devoid of any interrelation with other features of the disease.

[3] It was of great advantage to these researches that we could avail ourselves of the surgical skill and generous cooperation of two experienced members of this department. Prof. Ferdinand C. Lee collected toxiferous lymph from the thoracic duct of two sheep (nos. 6 and 7), in quantities that sufficed to establish the mode of distribution of the toxin between the blood and lymph, and Prof. Warfield M. Firor carried out the cross-circulation experiment on Sheep *A* and *B*.

[4] The well known dangers attendant upon surgical intervention in freeing viable spores enclosed in scar tissue or healed wounds, or in destroying granulating surfaces that act as a barrier to the further entrance of the toxin into the system, cannot be considered here. On the second point, see a recent paper by Matthew White which contains references to earlier experiments. In 1863 Billroth (8) made the following statement: "Ich glaube ferner beobachtet zu haben, dass

body, innumerable experiments have been made to determine the relative distribution of this substance—tetanus toxin—among the various organs and fluids of the body. Very naturally, much of the early work on this problem was of a qualitative character only, since no attempt was made to determine, with any degree of accuracy, how much of the toxin was injected at any given point, and how much of it was recoverable from the blood and lymph or other parts of the body. It was inevitable that the lack of precision and the absence of statistical evidence that characterized the work of that time should lead to confusion in respect to the true significance of the varying appearance in time and place of the several symptoms of the disease and of the relative order of their appearance. Deficiencies of this kind led earlier workers into the error of maintaining that five to seven times more of the toxin is required to kill an animal when it is injected into a vein than when it is injected subcutaneously. It has been contended, too, that even so small a quantity as the 1/400 part of the amount of toxin that did not, when injected subcutaneously, induce local tetanus in the muscles of a hind leg, will occasionally cause decided local tetanus when it is injected into the sciatic nerve instead of underneath the skin.[5] It was also too often taken for granted that the rapid disappearance of the toxin from the blood stream of rabbits that have received 10 or 12 lethal doses of it, holds true for other animals, inclusive of man. Other errors, some of which have been considered in our earlier papers, are likewise referable to a neglect of the above-named precautions. Nevertheless, many facts

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diejenigen Fälle, in welchen der Starrkrampf bei bereits granulirenden Wunden auftritt, chronischer und milder verlaufen, als diejenigen, in denen die Krankheit sich kurze Zeit nach der Verletzung entwickelt." (Page 337.)

While reading the proof of this paper, an article by Karl Halter (Ztschr. f. Hyg. u. Infektionskr., 1936, cxviii, 244) has come to our attention. Among other things this author has studied the action of tetanus toxin when injected into certain leg muscles of the rabbit that had previously been inflamed by means of abrin, jequiritol or vaccine virus. The effect of the toxin when injected into the highly inflamed muscles was studied in respect to local and ascending tetanus, the length of the incubation period, and the course of a fatal tetanus following large doses of the toxin.

[5] Loewi und Meyer: Arch. f. exper. Path. u. Pharmakol., 1908, Suppl.-Bd., S. 357.

of the greatest importance, quite aside from the manner of their interpretation, were brought to light by the investigators that undertook a study of tetanus during the last quarter of the preceding century, and it would be unjustifiable, on several counts, to ignore their factual contributions to our knowledge of the disease.

During the years 1859 to 1870 and even earlier, a number of leading surgeons and physicians expressed the opinion that tetanus must be classed with infectious diseases, such as rabies, and this at a time when the true cause of such diseases was still unknown. Richardson, Wells, Roser, Heiberg, Neudörfer, Billroth, Rose, and Arloing and Tripier, together with Frickenhaus, Strümpell and others of a later period named by Rosenbach, presented arguments in favor of this opinion, and some of them suggested that a poison similar to strychnin appeared in the infected wound, which might heal perfectly without any inflammatory reaction, as after the bite of a rabid dog, to quote the words of Billroth. Schultz, of Rostock, injected the blood serum and pus of a tetanic patient into the subcutaneous tissue of a dog; Billroth injected dogs intravenously with blood serum and the wound secretion from one of his patients; Arloing and Tripier injected such material from human cases into rabbits and dogs. These two experimenters also injected 200 cc. of blood from a horse with tetanus into the jugular vein of a normal horse. None of these authors succeeded in establishing the proof of the transmissibility of the disease from man to other animals, or from a tetanic horse to a healthy one. The reasons for their unfortunate failure to verify their sound conjectures are now clearly evident. Their negative results with such large test animals as rabbits and dogs may have been due either to the fact that they injected too small an amount of material or that they were dealing with tetanic patients and horses, whose blood contained little or none of the toxin, as often occurs when the quantity of toxin elaborated at the seat of infection is but little more than is needed to destroy life. In spite, however, of these negative results with inoculations of blood and purulent material, the belief persisted among continental physicians that tetanus is due to the noxious action of some type of "miasmatic or pyaemic poison," and it was suggested that the failure to transmit the disease to dogs and rabbits might be due to the fact that these animals are not naturally susceptible to it—an opinion that was later shown to be inadmissible, for the dog at least.

A wrong interpretation of their own work, however, led later investigators into new errors. There is an error that may claim precedence over all others in the field, one that has been blest with the approval of many who have tried to uphold it with anatomical arguments and "corroborative experiments," whose origin and tenacious vitality cannot be charged to faulty assays of the toxin, or to a lack

of statistical evidence. This error, so influential in the development of a false pathogeny of tetanus and scarcely less harmful as regards diphtheria and botulism,[6] is expressed in the belief that tetanus toxin is conveyed to the reactive cells of the central nervous system only by way of the motor fibers of peripheral nerves and that voluntary muscles cannot be tetanized by a direct action of the toxin upon them, but only when their controlling centers become hyperexcitable in consequence of the neural conveyance of the poison to them. We have considered this error at length in our preceding papers and shall not comment here on the steps that were taken by us to prove its falsity, the more so, as we are concerned in the present communication with problems of a different nature. The clarification of the pathogeny of tetanus is still hampered by the persistence of this dominant error, and of others of almost equal significance, in the confused collection of ideas on tetanus, that have been transmitted to our time. Among these last is the false belief, so widely held, that when

[6] Schübel (1923) does not hesitate to endow motor axons with the power of transporting the botulinus toxin away from the poisoned areas of the spinal cord, clear down to the motor end plates of voluntary muscles. He concludes that the resulting muscular paralysis can be explained only on the assumption of such a centrifugal conveyance either of the toxin itself, or of a poisonous metabolite that appears in the motor cells of the cord, consequent to the action of the toxin upon them.

Yuien, one of the many defenders of the theory "that the toxin spreads along the nerves and when it finally reaches the motor cells of the central nervous system has a powerful action like strychnine nitrate," came to the conclusion, on the basis of his intraneural injections with ferrocyanide solutions, "that the direction of lymphatic flow is centripetal in the motor nerves and centrifugal in the sensory nerves." The papers of this author have long been known to us but were not referred to in our previous papers because we believed that our experiments, as described in Paper III, had disproved the contention that tetanus toxin is ever carried to central neurons except it be forcibly injected into peripheral nerves. The very doubtful possibility, as it appeared to us, of a centrifugal lymph flow in sensory nerves had no immediate bearing on our own experiments that controverted the nerve transport theory. Yuien's work is also marred by the mistaken belief of other adherents of this theory that the toxin acts immediately upon the central neurons "like strychnine nitrate" the moment it reaches them; on the contrary, direct injection of a small amount of toxin into the anterior horns of the spinal cord, as will be proved in a later paper, is followed by a period of incubation of three or more days.

once a toxin finds entrance into the anterior horns, or at any point of the substance of the spinal cord, it moves freely in the tissue spaces in all directions, as for example, from the lumbar cord to the medulla or beyond, and in the opposite direction or across the cord, as the case may be. This error is the half-brother of the one that sponsored the centripetal carriage of toxin in peripheral motor nerves in the short space of two or more hours. The more or less rapid transmission of a toxin in the tissue spaces of the spinal cord to distal points is as impossible in the case of this organ, which is devoid of true lymphatics, as it is in the axis-cylinders of motor nerves. This poison, like others, can progress from one point of the spinal cord to another, aside from its transference by the blood stream, only by the exceedingly slow process of diffusion, as we shall endeavor to prove in our experiments on intraspinal injections that will be described in a later communication. The circulation constitutes the only mechanism of the spinal cord that can quickly distribute poisonous substances throughout the neural axis.

For more than a century, not to speak of earlier times, investigators have studied the comparative toxicity[7] of the many therapeutic agents employed in medicine and numberless other substances of organic and inorganic nature, whose therapeutic value is of a negative or doubtful character, but which, by virtue of their highly toxic properties, may become a menace to the health of human beings and the domestic animals. Some of the aspects of the many problems that arise in connection with the study of drugs and poisons of all kinds have been broadly considered in Paper I of our series (1).

New types of chemically induced disease were brought to light when it was proved that numerous varieties of pathogenic microörganisms elaborate chemical agents or toxins that play the dominant rôle in causing the symptoms and the cellular lesions of this class of diseases. The study of the comparative toxicity of these newly discovered toxins and their distribution among the organs and fluids of the body has always been seriously hampered by our deficient knowledge of their true character and chemical composition. We are not here in the

[7] See, for example, Flury und Zernik: Toxische und lethale Dosen für Gifte und Versuchstiere. Handb. biol. Arbeitsmethoden, T. 7, H. 7, 1928; and T. Sollmann: A Laboratory Guide in Pharmacology, Appendix H, 1917.

position of the pharmacologist, who can determine on the balance the exact weight of a pure crystalline drug or poison as a preliminary step to his investigations. The older and well tried methods had, therefore, to be somewhat modified in undertaking studies with the toxins and their antitoxins, such as those of tetanus, with which we are particularly concerned; but it must not be forgotten that comparative bio-assays were frequently made by physiologists and pharmacologists before the advent of the bacterial era, but not with any degree of statistical accuracy. As is well known, the bio-assay method of testing bacterial toxins and antitoxins, in accordance with their action on mice and guinea-pigs, was elaborated by Behring and his associates and their successors. Of late years, investigators have attained quantitative results of a considerable degree of accuracy by the application of statistical methods to their bio-assays. Statistical methods are now also widely employed by pharmacologists (using small animals as test objects) in establishing standard "units" for the efficacy of insulin and other hormones and of many important drugs, such as digitalis.

The fact that many drugs, toxins and other poisons have *a relatively uniform action on very large groups of healthy animals* has made it possible to establish, by such methods, fairly reliable units of their efficacy in terms of their action on small test-animals. This uniformity of action is particularly impressive when groups of white mice or guinea-pigs, of standard weight, are utilized in studies on the action of tetanus toxin. The manner in which we have used the bio-assay method in our own experiments will be described in the following section of this paper.

Of the many investigators who have studied tetanus from the experimental, as distinguished from the clinical point of view, may be named: Nicolaier, Brieger, Kitasato, Sanchez-Toledo and Veillon, Nissen, Vaillard and Vincent, Metchnikoff, Pestana, Stern, Brunner, Kallmeyer, Bruschettini, Kartulis, Quadu, Buschke and Oergel, Knorr, Decroly, Blumenthal, Bolton, Fisch and Walden, Marie and Morax, Meyer and his associates (Ransom, Loewi, Fröhlich and Halsey), McClintock and Hutchings, Permin, Teale and Embleton, Bieling and Gottschalk, Yasuyama and Schöbl. Some of these have examined for their content of toxin, the blood, lymph, cerebrospinal fluid

and urine, or one or more organs of human beings with tetanus; and all of them have made similar but more extended studies with experimental animals, that had received very large or small, but still fatal, doses of the toxin. In the decades 1880 to 1910 (a period of great activity in the studies relative to the fate of the toxin in the body) other names than those mentioned above may also be found; and it may be remarked that the findings of the writers of one nation, during this time, often merely corroborated those published by their contemporaries of another nation.

We shall concern ourselves more particularly in the present paper with the experiments of those authors who have made quantitative estimates of the toxin content of the blood, lymph, cerebrospinal fluid, spinal cord, voluntary muscles and other organs of experimental animals that had received known amounts of the toxin. In our own experiments we made use of guinea-pigs, dogs and rabbits; but also of sheep and horses, particularly because these animals are more highly responsive to tetanus than the dog or the rabbit and because their greater blood volume permits of the removal at suitable intervals of larger amounts of their blood without injuring them in any way.

We have endeavored, in the present paper (1) to learn to what extent the toxin accumulates in the blood and lymph of the different species of animals *pari passu* with the injection of increasing amounts of it; (2) to learn something about the relative ability of the different animal species to bind and dispose of the toxin in such a manner as to be no longer recoverable from their organs; (3) to estimate the bearing of the information obtained in these and in other experiments on a rational theory of tetanus.

II. EXPERIMENTAL METHODS

1. Preparation and storage of the toxin

Although it is customary to use in experimental work a dried, ammonium-sulphate-precipitated toxin, by reason of its stability over long periods of time, we decided to make use of known dilutions of the original toxic filtrates in the experiments reported in this paper and in our other studies on the pathogeny of the disease, for the following reasons. The precipitated material, as a rule, retains considerable

ammonium sulphate, a salt that would have been a disturbing factor, in our previously described intraneural as well as in our later intraspinal injections. The degree of purification effected in obtaining the toxin in the dry state is negligible; for various reasons, we could not at this time undertake chemical studies with the purpose of preparing a toxin of the highest purity. Such experiments as we have made with the ammonium-sulphate-containing precipitate have, like those of other investigators, never established any significant difference between the physiological action of this precipitate and that of the toxic filtrate from which it was obtained. Seeing that it was not suitable for our intraneural and intraspinal injections, and *because it was certainly advisable to assay our toxin every time it was injected*, we have made use of appropriate dilutions of very powerful toxic filtrates in the experiments presently to be described.

The strain of *clostridium tetani* utilized in the preparation of our toxin is that employed by the Mulford Biological Laboratories, in the production of their antitoxin, and we are indebted to this firm for kindly sending us a sub-culture.

Stock cultures of the organism were kept at room temperature in tubes containing a medium of chopped-beef, prepared according to the directions of Holman; the tubes were sealed with salvoline. A young, actively growing culture was used for seeding flasks of broth for the production of the toxin. This culture was always obtained by making three serial sub-cultures of the organism into tubes of fresh medium at twenty-four hour intervals; the last of the sub-cultures was then used for the seeding. The most potent lots of toxin were obtained by growing the organisms in Wadsworth broth, made up with 50 to 100 grams of Witte's peptone in place of the specified 10 grams of Berna's peptone. Incubation was continued for ten to twelve days at either 37°C. or 34°C. The cultures were now filtered through Zeiss filters, the filtrates transferred to test tubes in 25 to 50 cc. quantities, tested for sterility and kept in the dark at 3°C., except for the short intervals of time involved in the withdrawal of the toxin for bio-assays and experimental use. Under these conditions we have found that all of our six separate lots of toxin, no one of which was more than two months old, were remarkably stable, in that they gave no evidence of a determinable loss of toxicity, during the periods

in which they were employed. Further, there is present a minimal amount of toxoid, if any, in filtrates of this age, prepared and stored as described above. The method of assaying every toxic filtrate at the time it was injected into an animal gave us definite knowledge, in terms of guinea-pig units, of the amount of toxin that was injected in any given experiment.

2. Method of assaying the toxin; statistical treatment of the numerical data obtained in the bio-assays

In estimating the toxin content of our bacterial filtrates and that of the blood of the inoculated animals, guinea-pigs were made use of as test animals, with frequent parallel assays on mice weighing 20 grams. The guinea-pigs weighed 330 to 370 grams, were well nourished, but not of inbred stock, and remained free from intercurrent infections throughout the course of the test periods. Observations were made on each animal two or three times a day, and the development of the characteristic symptoms of tetanus carefully recorded; and since autopsies were made on all the animals that died, we can be reasonably certain as regards the number that succumbed to an uncomplicated tetanus poisoning. In rare instances, where the evidence of gross pathology suggested a concomitant bacterial infection, material from suspicious lesions was transferred to blood agar and infusion broth, for the detection of aerobes, and to tubes of chopped-meat or brain, for anaerobes. It was only in accordance with sound practice to make bacteriological tests in the case of those guinea-pigs that died within two or three days after the injection of 5 to 6 cc. of blood taken from sheep in the last stages of tetanus. One of our injected sheep, that had been subjected to anesthesia with ether for several hours, developed a terminal pasteurella septicemia and pneumonia along with tetanus. Many of the guinea-pigs, injected with the blood from this animal died from a frank pasteurella infection, sometimes before they showed symptoms of tetanus. Records of such a series were regarded as worthless, and were consequently discarded.

Preliminary studies were carried out to determine what influence, if any, the place of injection of the toxin into test guinea-pigs, whether into the subcutaneum or into muscles, or the volume of the injected

dose, which varied from 0.25 cc. to 5 or 6 cc., might have on the accuracy of the assays. Investigations were also made with respect to the use of three diluents, namely, 0.85 per cent NaCl solution, beef extract broth (pH 7.2), and whole blood. With a given diluent, variations in the total volume (0.25 to 5 or 6 cc.) of the injected dose were without effect upon the assay. It was disclosed, however, that with injections of equal doses of toxin in a given volume of blood, in 0.85 per cent NaCl solution, or in beef extract broth, the blood-toxin and broth-toxin injections were slightly more toxic than the saline. Moreover, it was soon found that the saline dilutions suffered a partial loss of toxicity on standing, and that marked irregularities were sometimes observed in the reaction of duplicate test animals to toxin-saline dilutions, while the response of the test animals was quite uniform to dilutions of the toxin that were made with broth or whole blood.[8] With each of the diluents, the most uniform results were obtained by intramuscular injections into the hind legs of animals. It will be seen, then, that in always assaying our toxic filtrates only after diluting them with blood taken from the same animals that were subsequently injected with such filtrates, we attained a desirable conformity between the original assays and those made with the toxiferous blood of the inoculated animals. Finally, it was found that the toxicity of a given blood-toxin mixture was the same, whether heparin or sodium citrate was used as the anticoagulant for the blood. Sodium citrate was, therefore, chosen as the more convenient of the two substances and was used in all of the experiments.

Guinea-pig median lethal dose (LD 50). In testing tetanus toxin, it has long been customary to state its efficacy in terms of the minimal lethal dose for guinea-pigs. By definition, a minimal lethal dose is that quantity of toxin that is just large enough to kill all of the guinea-pigs of a large population of standard weight in four or five days, with typical symptoms of tetanus. It is evident that the term

[8] The above experiments with diluents were made three years ago. In the meantime, Condrea and Peonaru have reported pronounced differences in the titers of both fresh and ammonium-sulphate-precipitated toxin with various diluents. Our observations are in entire agreement with their findings that toxins assayed with 0.9 per cent NaCl solution as the diluent show lower values than the same toxins assayed with 1 per cent peptone-water as the diluent.

"a large population" may have a different meaning for different observers and it is very doubtful, as has been pointed out by a number of writers, whether the term has ever been established with that degree of statistical accuracy that will enable us to speak of a "certainly minimal lethal dose."[9] It is recognized that a "minimal lethal dose," arrived at from an assay on a small number of animals, will by no means kill all the animals of a larger series within a selected time limit. The selection of four to five days as the survival time, instead of one that is longer or shorter, is justified by the inspection of fatality curves obtained by injecting graduated doses of toxic filtrates into many separate groups of guinea-pigs, from which it is apparent that the relation between dose and time of death is most nearly linear in the neighborhood of those amounts of toxin that kill the test animals in four to five days. With these facts in mind we have chosen, as our standard in this paper, that amount of toxin that kills fifty per cent of a given number of guinea-pigs, weighing 330 to 370 grams, in four to five days after injection, and have called this a *guinea-pig median lethal dose*, adopting the terminology suggested by Trevan.

Statistical method for determination of the guinea-pig median lethal dose (LD 50). We are greatly indebted to Prof. Lowell J. Reed, of the Department of Biostatistics of the School of Hygiene and Public Health of this University, for his kindness in examining our experimental data and for suggesting the following statistical method as suitable for their appraisal, in terms of an appropriate standard of toxicity—the guinea-pig median lethal dose referred to above. He has kindly permitted us to append the following brief exposition of his method:

"A reasonable definition of a lethal dose would be that dose for

[9] In 1927 Trevan studied and analyzed the errors involved in the determination of the "minimal lethal dose," or, the "minimal effective dose," of cocaine, digitalis, echitamine, dysentery toxin and insulin, and suggested that toxicity should be expressed by the term "median lethal dose," that is, that dose which kills 50 per cent of the animals. He calls especial attention to the error involved when a small number of animals is used, but points out that, after the characteristic curve has been set up, as few as ten animals may be used in the determination of the median lethal dose. In the assay of tetanus toxin used in our experiments, this characteristic curve for a 4 to 5 day survival time was well defined by preliminary assays.

which it is a fifty-fifty chance that an animal would die. This is represented in Diagram I, by dose A, the spread of this curve about this position giving the biological variation of the animals with respect to this lethal substance. The real problem, therefore, is the determination of the form of the curve so that the probability of death can be expressed as a function of the dose. The function may be symmetrical or skew, but whatever its form, we wish to know its statistical characteristics, such as centering value, variation, skewness, etc. The determination of this function is possible only if a large number of animals is observed, and in general this is impossible owing to experimental limitations. The practical problem is therefore the determination of the most probable position for the point A, and of some measure of the probable error of this point when the number of animals observed is small.

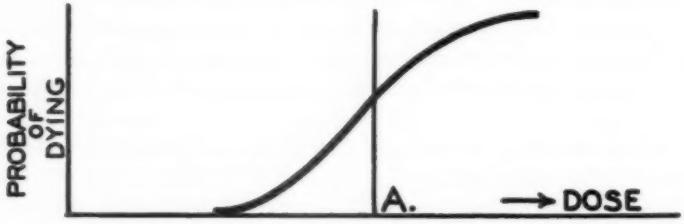

DIAGRAM I

"To solve this problem we shall use the formula of inverse probability that says if an event has been observed to happen n times and fail m times, the probability that it will occur on the next trial is:

$$\frac{n + 1}{n + m + 2}.$$

"(a) In addition to this rule, we shall assume that if a particular animal died at dose x, it would also have died at dose $x + a$; (b) that if an animal lives after receiving dose x, that animal would have lived at any dose $x - a$. These two statements are certainly in keeping with the previously stated concept of continuity with regard to lethal dose.

"Applying these assumptions to each dose, x, we can get n, the

number of deaths at the dose x and under, and m, the number alive at this dose x or over. The formula

$$p = \frac{n + 1}{n + m + 2}$$

gives the probability that a death will occur at dose x. Although the p thus determined is only an approximate measure of the proportion of animals that will die at dose x, it is as accurate a determination as the data warrant.

"Plotting p against the dosage, we have the representation of the degree of variability of p with dose, as well as it is indicated by our sample.

"The curve used to represent the values of p for different doses might be any one of several, but it is natural to take the integral curve of the normal probability curve. In a problem that has such wide variation as the one under consideration, the particular form of curve used is not a matter of great importance. Assuming, then, that the values are on the integral of a normal probability curve, the values of p should plot in linear form on probability paper. The problem may thus be solved graphically by plotting on probability paper, drawing a straight line through the points and reading the value of A at the position where this line crosses the 50% line of the probability paper. We might adopt some method of fitting this line, but we are hardly justified in this, owing to the scantiness of the material. There are several points to be remembered in drawing this line through the points. The most important thing is that in the determination of A the values of p immediately above and below $\frac{1}{2}$ are the ones of greatest weight. The straight line should, therefore, be based principally on the central values.

"To find the variation, we measure the difference in dose between p .50 and p .25. This value measures the probable error (P. E.) of an individual as given by observed points. The probable error of the lethal dose would be approximately equal to the probable error of an individual divided by $\sqrt{n' - 1}$, where n' is the number of animals that enter into the determination of the straight line. It should be noted that n' is not necessarily the total number of animals entering into the determination, for we may have animals tested at high or low doses that give us very little information."

Manner of carrying out experiments to meet the requirements of the median lethal dose. It will be apparent to the reader that there are three important requisites for a successful application of the selected statistical method. In the first place, a range of dosage is required that is sufficiently wide to insure that deaths occur in some groups of the test animals while in other groups the animals remain alive; (2) the doses must be graduated so that at least two of them fall slightly below and two slightly above the median lethal dose; (3) no less than two animals should be used for each dose, in order to minimize, to some extent, fluctuation due to individual variation in response to the toxin.

All of these requirements have been fulfilled in our experiments. The *preliminary* assays of the toxic filtrates gave us information in regard to the range of dosage required to kill guinea-pigs in four to five days. Then, guided by these preliminary assays, we could easily select six to eight doses of a suitably diluted toxic filtrate, none of which doses varied too widely in toxicity from that of the standard lethal dose; each of these varying six to eight doses was then tested on a pair of guinea-pigs. A greater degree of accuracy would, no doubt, have been attained in these assays of our toxic filtrates, had we used three or four animals in testing each dose.

In assaying the blood of inoculated animals for its toxin content we injected it into test pigs in varying quantities in order to anticipate possibly wide fluctuations in the toxicity of the blood. Actually, our injections of the toxic blood were limited in range to five or six different amounts of it; each of these amounts was tested on a pair of guinea-pigs. When the time of death of each group was recorded, and the data obtained in the course of the assays were analyzed, it was found that the injected quantities of toxic blood were evenly distributed above and below the median lethal dose, and that this standard unit of toxicity could therefore be arrived at with a fair degree of precision.

It is probably not amiss at this point to call attention to the practical limitations to the number of animals that can be employed in a study of this type. In the actual performance of every experiment, in which the toxin content of the blood of the inoculated animals was assayed for several consecutive days, from five to six dozen guinea-

pigs were usually required; in one case (Horse no. 2), in order to complete the experiment, sixteen dozen test animals were employed.

By application of this method of statistical analysis to the bioassays made with a toxic filtrate prior to injection and with the blood of an animal inoculated with it, it is possible to determine the quantity, of either the filtrate or the blood, that contains one guinea-pig median lethal dose. Knowing the total quantity of toxic filtrate injected into an animal, we are enabled to calculate the total number of guinea-pig median lethal doses administered; if the blood volume of the inoculated animal is known, it is also possible to estimate the number of guinea-

TABLE I

Assay of toxin no. 7 on guinea-pigs

Dilutions of toxin made with citrated sheep's blood. One cc. of each dilution was injected intramuscularly into each of two animals.

DILUTION	ANIMALS DEAD IN 5 DAYS	ANIMALS ALIVE IN 5 DAYS	n	m	p
1–6,000	2	0	7	0	0.89
1–8,000	2	0	5	0	0.86
1–9,000	2	0	3	0	0.80
1–10,000	0	2	1	2	0.40
1–12,000	1	1	1	3	0.33
1–14,000	0	2	0	5	0.14
1–16,000	0	2	0	7	0.11
Controls*	0	2			

Toxin no. 7 = 10,700 ± 500 guinea-pig median lethal doses per cc.

* Control animals. Two animals injected with 5 cc. each of normal citrated sheep's blood.

pig median lethal doses that can be recovered from all of its blood at any given time. The number of such standard units of toxin found in the blood constitutes a proportionate part of the number that was injected, and may therefore be expressed in percental terms.

Blood volume of sheep. After consulting the available literature we decided to use the very conservative basic value of 66 cc. of blood per kilogram of the body weight of *shorn* sheep, in estimating their blood volume. If both terms of this ratio were expressed gravimetrically, it would read 69.3 grams of blood per kilogram of body weight, if one takes 1.05 for the specific gravity of the blood of sheep. This

way of stating the ratio is preferred by many, but for our purpose the former is preferable for the reason that our assays were made with a definite volume of blood and lymph, rather than with a specified weight, and we were thus able, without further computation, to express our final results in percental amounts of toxin in the total volume of the blood and lymph of an animal. The blood volume of sheep is usually given at a higher figure than that employed by us, and it may well be that we should have made use of the usually accepted ratio of blood volume to body weight, 1:12 to 1:13, as given by Paechtner; or that reported by Schulz, which was based on an analysis of the results obtained by various authors in their studies of the blood volume of the domestic animals, from which it was deduced that the blood volume of unshorn sheep, in cubic centimeters, is 8.01 per cent of their body weight.

3. Details of one experiment with sheep to show how the quantitative data were gathered, analyzed and interpreted

A detailed account of the data obtained in one experiment and the method of analysis of this material is presented below. Daily observations on the experimental animal are shown in the protocol. In Table I the details of the assay of the toxin are given. After calculating the value of p, we plotted the obtained figures against the various doses diluted to thousands, as shown in Graph I, and, after drawing a straight line through the obtained points, we read off the guinea-pig median lethal dose. One cubic centimeter of the toxin was thus found to contain 10,700 guinea-pig median lethal doses. Since 2.2 cc. of a 1–10 dilution of the toxin was injected into the sheep, we estimate that it had received 2,354 guinea-pig median lethal doses. We have recorded in Table II the experimental data on the daily assays of the toxicity of the sheep's blood, immediately following withdrawal and the addition of sodium citrate. The citrated blood was injected in 0.25, 0.5, 1.0, 2.0 and 3.0 cc. quantities into each of two guinea-pigs, and the time of death of the animals recorded. Again, after calculating the values of p, the obtained figures were plotted against the injected doses in cubic centimeters of blood, as shown in the corresponding graphs (II to V), and the amount of blood representing a guinea-pig median lethal dose read off. Then, knowing

TABLE II

Assay of toxicity of blood of sheep no. 4, drawn at regular intervals following the intravenous injection of 2,354 guinea-pig median lethal doses

HOURS AFTER INJECTION	DOSES OF BLOOD	ANIMALS DEAD IN 5 DAYS	ANIMALS ALIVE IN 5 DAYS	s	ss	p	RESULTS		
							Volume of blood containing 1 guinea-pig median lethal dose	P.E.	Calculated percentage of injected toxin that remained in the blood
	cc.						*cc.*	*cc.*	*per cent*
1	3.00	2	0	6	0	0.88	0.8	±0.2	120
	2.00	2	0	4	0	0.83			
	1.00	2	0	2	0	0.75			
	0.50	0	2	0	2	0.25			
	0.25	0	2	0	4	0.16			
7	3.00	2	0	4	0	0.83	1.5	±0.3	62
	2.00	2	0	2	0	0.75			
	1.00	0	2	0	2	0.25			
	0.50	0	2	0	4	0.16			
	0.25	0	2	0	6	0.13			
24	3.00	2	0	3	0	0.80	1.9	±0.3	49
	2.00	1	1	1	1	0.50			
	1.00	0	2	0	3	0.20			
	0.50	0	2	0	5	0.14			
	0.25	0	2	0	7	0.11			
48	3.00	2	0	4	0	0.83	1.5	±0.3	62
	2.00	2	0	2	0	0.75			
	1.00	0	2	0	2	0.25			
	0.50	0	2	0	4	0.16			
	0.25	0	2	0	6	0.13			
72	3.00	2	0	3	0	0.80	2.0	±0.3	46
	2.00	1	1	1	1	0.50			
	1.00	0	2	0	3	0.20			
	0.50	0	2	0	5	0.14			
	0.25	0	2	0	7	0.11			
96	3.00	2	0	2	0	0.75	2.0	±0.3	46
	2.00	0	2	0	2	0.25			
	1.00	0	2	0	4	0.16			
	0.50	0	2	0	6	0.13			
	0.25	0	2	0	8	0.10			
120	3.00	1	1				Slightly more than 3.0		Approximately 31
	2.00	0	2						
	1.00	0	2						
	0.50	0	2						
	0.25	0	2						

the blood volume of the sheep, we were enabled to determine the total number of guinea-pig median lethal doses of toxin circulating in its blood at any given time. The relative amounts of the originally injected toxin that were recovered from the blood, throughout the period of five days, is given in percentages in the last column of Table II. The probable error in the determination of the quantity of blood representing one guinea-pig median lethal dose is also given.

PROTOCOL OF SHEEP NO. 4

May 9, 1933. A ewe lamb, 33.0 kilograms. Estimated blood volume 2178 cc. At 8.45 A.M., 50 cc. of blood were removed from the left jugular vein and used for dilution and assay of the toxin.

9.10 A.M.: A 1–10 dilution of toxin no. 7 was made with sheep's blood; 2.2 cc. of this solution were injected slowly into the right jugular vein. This represents 2354 ± 107 guinea-pig median L.D.'s or 71.3 ± 3.2 guinea-pig median L.D.'s per kilo.

10.10 A.M.: 12 cc. of blood drawn from left jugular vein for assay. Ten guinea-pigs were injected.

May 10. Animal normal. Blood drawn at 10 A.M. (15 cc.). Ten guinea-pigs were injected.

May 11. Animal normal. Blood drawn at 10 A.M. (15 cc.). Ten guinea-pigs were injected.

May 12. Rapid respiration. Slight degree of trismus. Gait normal. Blood drawn at 10 A.M. (15 cc.). Ten guinea-pigs were injected.

May 13. Pronounced trismus. Animal refused food. Muscular stiffness, particularly in hind legs, though animal can still walk. Rapid respiration Blood drawn at 10 A.M. (15 cc.). Ten guinea-pigs were injected.

May 14. Animal prone; legs stiff and extended. Extreme salivation; trismus; central reflex symptoms of tetanus. Blood drawn at 10 A.M. (15 cc.). Ten guinea-pigs were injected.

May 15. Animal dead at 9 A.M. Autopsy, by Dr. Remsen of the Department of Pathology of this University, disclosed no gross pathological changes.

III. EXPOSITORY AND CRITICAL COMMENTS ON THE RESULTS OBTAINED IN OUR EXPERIMENTS WITH SHEEP AND HORSES

A study of the blood and lymph of animals that have been inoculated with the toxin of tetanus yields, in our opinion, information of the greatest value in respect to several important phases of the disease. The results of a study of this character are of especial significance for the pathogeny of human tetanus, when they are obtained in experi-

ments on animals, such as the horse, whose responsiveness to the toxin approaches or equals that of man; but even when the study is confined to animals that are somewhat less sensitive to it, such as sheep, the results appear to be of equal significance. An examination of our findings makes it plain why they can be set forth in explicit terms; but these findings also contain implicitly certain quite unambiguous ideas that acquire an explicit and definite character when they are considered in connection with either local or central motor tetanus, or with these conjointly.

Let us consider here the explicit or direct results of our blood and lymph studies as they have been brought to light in the course of bio-assays in which the blood of sheep was assayed after inoculating them with small but fatal amounts of the toxin.

Injected toxin quantitatively recoverable from the blood and lymph, with the exception of a portion practically equivalent to a single lethal dose. Two of our sheep (nos. 2 and 13 of Table III) were injected with small but still supralethal doses of the toxin. The former received 10 guinea-pig median lethal doses per kilogram of body weight by a single intramuscular injection into the shoulder, while the latter received 15 such guinea-pig doses per kilogram of body weight by multiple intramuscular injections at 19 separate points into each of the forelegs and at 22 separate points into each of the hind legs. Considerable amounts of the toxin appeared in the blood of both animals. The blood of Sheep no. 2, that was taken from the second to the fifth day after injection, killed guinea-pigs of standard weight in from 6 to 10 days. Symptoms of descending tetanus appeared in this sheep on the fifth day after the injection and the animal died on the eighth day. The blood of Sheep no. 13, that had received 5 units of toxin per kilogram more than Sheep no. 2, appeared to have less of it in its blood than Sheep no. 2. Blood taken during the first three days subsequent to injection of the toxin killed 4 out of 9 guinea-pigs, that had been inoculated with 6 cc. portions of it, in seven to twelve days; such large quantities as 6 cc. of the serum of this sheep, taken later than the third day, caused only symptoms of tetanus in guinea-pigs but no deaths.

The reason for the lower blood toxin content of Sheep no. 13 lies in the manner in which the toxin was administered. As stated in the

TABLE III

Showing the difficulty of assaying the amount of toxin in the blood of sheep that have received 10 and 15 guinea-pig median lethal doses per kilogram, and of two horses that received 4 and 5 doses, respectively

DATE	ANIMAL	WEIGHT	ROUTE OF INJECTION	DOSE PER KILOGRAM IN GUINEA-PIG MEDIAN L.D.'s	TOTAL DOSE IN GUINEA-PIG MEDIAN L.D.'s	RESULTS ON TOXICITY OF THE BLOOD	REMARKS
1933		*kgm.*					
Jan. 31	Sheep no. 2	26.0	Single intramuscular injection into the shoulder	10	260	From 2 to 5 days after injection, 3 to 4 cc. of blood killed guinea-pigs in 6 to 10 days. Data were insufficient for percentage calculations	Symptoms of descending tetanus appeared on 5th day after injection; animal dead on 8th day
1934							
May 30	Sheep no. 13	56.1	Multiple intramuscular injections at 19 points into each of the fore legs and at 22 points into each of the hind legs	15	872	From 1 to 3 days after injection, 6 cc. portions of blood killed 4 out of 9 injected guinea-pigs in 7 to 12 days. Thereafter, 6 cc. of serum caused symptoms of tetanus but no deaths in guinea-pigs. Data were insufficient for percentage calculations	Very marked symptoms of local tetanus in four legs on 4th day after injection; animal prone on 5th day with symptoms of descending tetanus (trismus) and central manifestations of the disease; etherized to death on 8th day, in the course of a study on the effect of ether in relaxing the rigid legs

1933							
Feb. 17	Horse no. 1 Mare, aged 11 years; in perfect health, according to the veterinary surgeon, Dr. J. B. Byrnes	436	Single superficial intramuscular injection into the shoulder	5	2,180	Symptoms of tetanus without deaths were produced with various quantities of blood drawn at regular intervals after the inoculation of the horse. After 5 hours 2 cc. portions caused rigidity of the hind legs of test animals injected intramuscularly into the thigh; after 1, 2 and 3 days, respectively, 1 cc. portions; after 4 and 5 days, 2 cc. portions. On the 6th day blood taken one-half hour after the animal was shot failed to produce symptoms when injected into the hind legs of guinea-pigs in 3 cc. portions; larger quantities were not tested	First symptoms of descending tetanus appeared on 5th day after injection; on the morning of the 6th day, animal lay prone with convulsive attacks of such violence that the sides of the stall were kicked out. When these attacks had subsided sufficiently to allow of approach, the animal was shot. Throughout the entire period, 3 or 4 cc. of urine were tested each day on guinea-pigs; the test animals remained normal
Mar. 21	Horse no. 2 Riding horse, mare, 6 yrs. old; in perfect health, according to Dr. J. B. Byrnes	390	Single subcutaneous injection into shoulder	4	1,560	Injections of 6 cc. portions of blood drawn daily produced no symptoms of tetanus in guinea-pigs. Serum of blood taken on the 6th, 7th, 8th, 12th and 13th days, respectively, were also injected into pigs, in 6 cc. portions; the animals showed no symptoms of tetanus	First symptoms of descending tetanus appeared on 8th day (difficulty in eating); on 9th day, pronounced trismus, tail arched, slight stiffness in gait; on 10th day, symptoms much the same except for increase in degree of trismus; on 11th day, profuse salivation, complete trismus; on 12th day, profuse salivation, rapid respiration. On 13th day, animal suddenly fell to ground and could not get up; hind legs spread apart; neck arched; profuse sweating and salivation; R.T. 106.6°; blood taken for bio-assay; animal was shot

protocols, the total quantity of the toxin was injected intramuscularly, in divided doses, at 19 separate points into each of the forelegs and at 22 points into each of the hind legs. As a result of this mode of injection, very marked symptoms of local tetanus appeared in the four legs of the animal on the fourth day after injection. On the fifth day the animal lay prone, and not until this day did symptoms of descending tetanus (trismus) and central manifestation of the disease make their appearance. On the eighth day, when death was already imminent, the animal died on the operating table from an overdose of ether, in the course of a study on the action of this anesthetic in relaxing the rigidly contracted legs. It would appear that the intramuscular injection of the toxin at 82 separate points of the four legs (Sheep no. 13) was followed by a greater local retention of the toxin in the many injected areas, in comparison with what took place in the other sheep (no. 2) that had received the entire amount of its toxin at a single intramuscular injection into the shoulder. This fixation of a larger amount of toxin by the tissues of Sheep no. 13, in comparison with that fixed by the tissues of Sheep no. 2, is proved by the fact that the blood of the former animal contained relatively less toxin than that of the latter; this, in spite of its having received 50 per cent more of the toxin.

It is apparent that it was not easy to assay with the desired accuracy the blood of Sheep nos. 2 and 13, although both received a somewhat larger quantity of toxin than was needed to kill them. Had our method of bio-assay been based on a survival time of seven to nine days, instead of the usual four to five days for test pigs, we should have been able to determine the amount of toxin present in the blood of the two sheep, such as it was, with the same degree of accuracy as in the experiments of Table IV. Nevertheless, the bio-assays of the blood of these two sheep (each of which had received more of the toxin than was needed to kill it) demonstrate how rapidly the toxin content of the blood diminishes as we go from higher to lower doses and approach the true minimal lethal dose of the toxin—a dose not easily determinable with small groups of animals.

Toxin not recoverable from the blood of sheep after a sublethal dose. We have recently (July, 1936) made an additional experiment in which a two-year old and shorn sheep (no. 14), weighing 50 kilo-

grams, received by subcutaneous injection into the tail a quantity of toxic filtrate containing a total of 150 guinea-pig units (LD50's), or, three of such units per kilogram, as compared with the 10 units per kilogram injected into Sheep no. 2. The toxin content of the blood of Sheep no. 2, when compared with that found in the case of others that had received larger doses of the toxin, showed that it fell off rapidly when the smaller dose was injected. We were interested to learn whether we would still find toxin in the blood of a sheep that had received only a single lethal dose or less. It was found that the 3 guinea-pig units (LD50's) per kilogram, that had been injected into Sheep no. 14, constituted only a sublethal dose. The serum of its blood was assayed for the first time five hours after injection of the toxin and once a day for 12 days thereafter. Varying amounts of this serum up to a maximum of 10 cc. were injected into a series of guinea-pigs, as in the other experiments with sheep. None of these injections of serum induced in the guinea-pigs any symptoms of either local or generalized tetanus. The sheep whose blood was thus shown to contain no detectable amount of the subcutaneously injected toxin, never showed the slightest symptoms of tetanus at any time during the 21 days it was under observation; the animal consumed large amounts of fodder and drank freely during this time, and although we neglected to weigh it before disposing of it, neither we nor our attendants entertained any doubts as to its excellent condition. While we failed with our injections into sheep to hit upon the exact amount of toxin that just suffices to kill (irrespective of a time limit for death), we nevertheless feel justified in believing that, as in the case of the horse and other highly susceptible animals, the blood stream of the sheep harbors no detectable part of a single lethal dose of the poison.

Intravenous injection. The intravenous injections into these animals of amounts of toxin that were carefully assayed in terms of guinea-pig median lethal dose units (LD50's)—amounts which might be roughly estimated as varying from 2 to 10 "minimal lethal doses" for the sheep—gave results that we deem to be of great interest, when they are compared with those obtained with intramuscular and subcutaneous injections. We shall take up in detail only the experiment with the intravenously injected Sheep no. 4 (Table IV). This animal,

TABLE

Showing the large amount of toxin circulating for days in the blood of sheep injected, by var

DATE	SHEEP NUMBER	WEIGHT	CALCULATED BLOOD VOLUME	ROUTE OF INJECTION	DOSE PER KILOGRAM IN GUINEA-PIG MEDIAN L.D.'S	TOTAL DOSE IN GUINEA-PIG MEDIAN L.D.'S	VOLUME OF TOXIC BLOOD MEDIAN			
							1 hr.	7 hrs.	24 hrs.	48 h
1933		*kgm.*	*cc.*				*cc.*	*cc.*	*cc.*	*cc.*
May 9	4	33.0	2,178	Intravenous	71.3	2,354	0.8	1.5	1.9	1.
Apr. 29	3	34.0	2,244	Single intramuscular into shoulder	61.9	2,116			2.0	2.
Jun. 20	6	35.3	2,330	Single intramuscular into shoulder	45.9	1,620			2.5	3.1 blood 2.5 lymph
Jul. 18	7	45.4	2,996	Intravenous	47.2	2,142			2.0	2.5 venou 2.8 lymph 3.4 arteri
1934										
Feb. 13	12	41.1	2,713	Intravenous	64.8	2,662				3.1
1933										
Jan. 17	1	50.4		Subcutaneous injections at various points of the tail	25	1,038				
										Cross-circulation
1933										
Oct. 10	A (donor)	50.5	3,333	Intravenous	92.6	4,680			1.7	1.7 before fusion 2.3 after fusion
Oct. 12	B (recipient)	45.1	2,977	Under ether anaesthesia Dr. Firor made a cross-circulation between the femoral artery and vein of each of the two animals for 35 minutes	24.4	1,102	2.7		5.0+	6.0+

TABLE IV

ected, by various routes, with 24 to 92 guinea-pig median lethal doses per kilogram of body weight

OF TOXIC BLOOD CONTAINING ONE GUINEA-PIG MEDIAN LETHAL DOSE					CALCULATED PERCENTAGE OF INJECTED TOXIN CIRCULATING IN BLOOD OF SHEEP					
48 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.	1 hr.	7 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1.5	2.0	2.0	3.0	3.0	120	62	49	62	46	46	31 (approx.)
2.2	2.2	2.2	2.2	2.2			53	48	48	48	48
3.1 blood 2.5 lymph							57	46 blood 57 lymph			
2.5 venous blood 2.8 lymph 3.4 arterial blood	3.5	4.0+					70	56 venous blood 50 lymph 41 arterial blood	40	Slightly less than 35	
3.1								34			
							45	45	45	45	45 (approx.)
-circulation experiment											
1.7 before transfusion 2.3 after transfusion	3.0	2.6					42	42 before transfusion 31 after transfusion	24	27	
6.0+	6.0+	6.0+			24		Slightly less than 13	Slightly less than 11	Less than 11	Pasteurella infection	

COMMENTS ON EXPERIMENTS OF TABLE IV

Sheep No. 4: First symptoms of descending tetanus (involvement of the jaw muscles) appeared on 3rd day after injection; fourth day stiffness of hind legs, rapid respiration; animal prone on 5th day, with symptoms of central tetanus, which appeared in marked form only on this day; found dead on 6th day.

Sheep No. 3: Very marked symptoms of descending tetanus appeared on 4th day after injection; animal prone on 6th day, with central manifestations of the disease; dead on 7th day.

Sheep No. 6: The thoracic duct was cannulated, under ether anesthesia, by Dr. Lee 40 hours after the injection, and lymph collected for assay; blood for assay was drawn before operation. Very marked symptoms of descending tetanus (trismus) appeared on 4th day; animal dead on 5th day. Autopsy by Dr. Remsen of the Department of Pathology showed that pneumonia had developed. For the calculation of percentage of the injected toxin in the lymph, we have assumed that the total amount of lymph in the entire lymphatic system can be conservatively estimated as equal to that of the blood.

Sheep No. 7: Under ether anesthesia, lymph was collected from the thoracic duct by Dr. Lee 48 hours after the injection of the toxin. Venous blood for assay was drawn one hour before the operation, and arterial blood during the operation. The first symptoms of descending tetanus appeared on the 3rd day; animal died exactly 5 days after the injection. The middle portion of the right sciatic nerve, weighing 1½ gms., was removed, ground up with saline and injected intramuscularly into the right hind leg of a guinea-pig; guinea-pig remained normal.

Sheep No. 12: This experiment must be regarded as incomplete, since we have only one assay on the toxin content of the blood. On the 3rd day after the injection of the toxin, during the abstraction of a large volume of blood, for purposes which we need not here state, the animal unfortunately succumbed to ether anesthesia. Immediately before the anesthesia, 15 cc. of spinal fluid were collected by Dr. Firor. Guinea-pigs injected intramuscularly with 5 cc. portions of it remained normal. The animal showed no symptoms of tetanus on this day.

Sheep No. 1: On the 3rd day very marked symptoms of descending tetanus (pronounced trismus) appeared; animal unable to stand; central manifestations of tetanus also present. Animal dead on 7th day.

Injections into guinea-pigs of 3 cc. portions of blood drawn daily killed the animals in 5 to 7 days, but since only one animal was injected with each dose, the LD50 could not be estimated. Our data indicate, however, that at least 45 per cent of the injected toxin circulated in the blood of the sheep from the 1st to the 5th day after the injection.

Sheep A: On the 2nd day after the injection, a cross-circulation was made with a normal sheep (Sheep B). Blood was drawn for assay five hours before the cross-circulation was established, and again less than one hour after the cross-circulation was discontinued. Marked symptoms of descending tetanus (trismus, salivation) and stiffness of hind legs appeared on 4th day after injection; animal dead on 5th day.

Sheep B: This animal received toxic blood from Sheep A intravenously, by means of cross-circulation, for 35 minutes. Blood for assay was drawn within 20 minutes after the cross-circulation was discontinued. After taking into account the body weight and blood volume of Sheep B, it was estimated, from the assay, that the animal received 24 per cent of the toxin originally injected into Sheep A, or 24.4 guinea-pig median lethal doses per kilo, a quantity easily lethal for the animal. (Compare with Sheep Nos. 1, 2 and 13.) On 2nd day after transfusion, animal was able to walk, but on 3rd day showed some stiffness in hind legs; animal prone with marked symptoms of descending tetanus (trismus) on 5th day; dead on 6th day. Autopsy revealed definite pneumonia. Guinea-pigs injected with blood taken on the 4th and 5th days, died from a pasteurella infection.

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weighing 33 kilograms, with a calculated blood volume of 2,178 cc., received a total of 2,354 guinea-pig units (LD50's) of the toxin. At the end of one hour after inoculating this amount of the toxin into the jugular vein, the blood, as assayed by tests on guinea-pigs, contained 120 per cent of the toxin—a figure that involves the statistical error referred to in Section II. At this time, then, all of the injected toxin was apparently still present in the blood and only a relatively small amount of it could have passed over into the lymphatic system.

In the next seven hours the toxin content of the blood of this sheep fell to 62 per cent of the injected quantity; after 24 hours, it was 49 per cent; after 48 hours, 62 per cent; after 72 hours, 46 per cent; after 96 hours, 46 per cent; and after 120 hours (or 5 days), 31 per cent. The first light symptoms of descending tetanus appeared on the third day after the injection, at a time, therefore, when the amount of toxin in both blood and lymph still amounted to perhaps 90 per cent of that injected. On the fifth day the animal lay prone on its side with the most marked symptoms of central tetanus. It was found dead on the morning of the sixth day. From the twenty-fourth hour on, the toxin of the lymph was in equilibrium with that of the blood, in the sense that equal volumes of the two fluids contained equal amounts of the toxin, as will be shown in the experiments with Sheep nos. 6 and 7 (Table IV).

The greater part of the injected toxin is recoverable from these two fluids, as will now be shown; the fraction so firmly bound as to evade detection is equivalent to about a lethal dose.

Relative amounts of toxin in blood and lymph. This question was

studied in experiments with Sheep nos. 6 and 7, each of which had been given approximately the same number of toxin units per kilogram of body weight, but by different pathways. No. 6 received the toxin by intramuscular injection at a single point into the shoulder, and no. 7 received it by the jugular vein. Twenty-four hours later the toxin content of the venous blood of no. 6 was found to be 57 per cent of what had been injected, and after 48 hours, 46 per cent, while the toxin content of the lymph at this time assayed 57 per cent of the amount originally injected. The toxin content of the venous blood of no. 7, the intravenously injected sheep, tested twenty-four hours after injection, was found to be 70 per cent of that originally injected; after 48 hours its venous blood assayed 56 per cent, the carotid blood 41 per cent, and the lymph from the thoracic duct 50 per cent. The blood and lymph streams of the two sheep were, therefore, certainly in equilibrium in respect to their toxin content as early as 30 to 48 hours after the injection, although one of the two had received the toxin by way of its muscles and the other by way of the jugular vein. If we make the permissible and conservative assumption, that the volume of lymph in the closed lymphatic system is equal to that of the entire blood, our bio-assays would seem to prove that more than 90 per cent of the injected toxin was present in assayable form in the blood-lymph systems of the two sheep and that it continued at this level for several days following the injection. The results of experiments made with Sheep nos. 3 and 4, that are recorded in Table IV, give added support to this conclusion.

We may therefore regard it as proved that when such quantities of toxin as were utilized in our experiments with sheep are injected, either into the muscles or directly into the blood stream, the toxin will be found to be present 48 hours thereafter, in equal and large amounts in equal volumes of the blood and lymph, and that it will maintain itself in these two fluids at a fairly steady level almost to the time of death. It is also apparent that when the toxin is introduced at some peripheral point of the body, it *rises* to this steady level by starting from a point of zero-absorption by the blood, but that when the entire amount of it is injected in the course of a few seconds into the blood stream, it *drops* from a 100 per cent level, and then reaches exactly the same permanent lower level in both blood and lymph, and after the same time interval, as in the former instance. The disposi-

tion of the toxin following its absorption from a peripheral point of the body can be plotted as an ascending curve, and that following its intravenous injection as a descending one; the single point at which the two curves intersect marks the time when the equilibrium referred to has been attained in the two cases, and also denotes its numerical value.

Cross-circulation in sheep. The cross-circulation experiment of Table IV presents certain points of interest. Sheep *A*, the donor animal, received intravenously a somewhat larger dose of toxin than had been introduced into the five other sheep referred to in Table IV, namely 90.6 guinea-pig median lethal doses per kilogram of its body weight. Twenty-four hours after the injection the content of toxin in the venous blood of this animal was 42 per cent of the injected quantity. Forty-eight hours after the injection it was still 42 per cent, and the lymph undoubtedly had the same amount in an equal volume. Five hours after this forty-eight hour blood was drawn a cross-circulation was established between Sheep *A*, and a second normal sheep, *B*. The two etherized animals were so placed that the femoral artery of *A* could be connected with the femoral vein of *B*, the recipient, and the femoral artery of the latter could be connected with the femoral vein of the former, or donor animal. The operation lasted about four hours and was unduly prolonged because it had to be interrupted while new connecting cannulae of the proper length and curvature were being made. The cross-circulation was kept up for 35 minutes. Blood was drawn from the jugular vein of each animal 15 minutes after the cross-circulation was discontinued. Table IV shows that the toxin content of the blood of the donor fell from 42 per cent to 31 per cent during the cross-circulation. In the following 24 hours it still assayed at 24 per cent, and after another 48 hours at 27 per cent. These figures are in good agreement with each other, and show that the blood toxin content of the donor, *A*, was maintained at a steady lower level after the loss of toxin by transfusion to the recipient, *B*. It was our purpose originally to make two more cross-circulation experiments with Sheep *A* as the donor, but we desisted from the attempt to do so in view of the well known fact that sheep easily develop pasteurella septicemia and pneumonia following long periods of anesthesia with ether.

Fifteen minutes after the cross-circulation was discontinued the

blood of the recipient, *B*, which of course was entirely free from toxin to begin with, showed that it contained 24 per cent of the toxin originally injected into the donor, *A*. The blood volume of Sheep *B* was taken to be 2,977 cc. Account was taken of the small difference of 366 cc. in the blood volume of the two animals. Twenty-four hours later the blood toxin content of the recipient, *B*, had fallen to 13 per cent of that originally injected into the donor. This remanent 13 per cent in *B*'s blood is equivalent to a drop of nearly 50 per cent of the toxin that was present in this blood 15 minutes after the conclusion of the cross-circulation. This drop in twenty-four hours is fully accounted for by the early removal and fixation of a fatal fraction of the toxin received from *A*, and by the escape of another fraction into the lymphatic system. During the following two days the toxin content of the blood of this sheep remained at practically the same level, namely, 11 per cent. On the third day after the cross-circulation, the first symptoms of descending tetanus appeared. On the fifth and sixth days, the blood assay with guinea-pigs began to be unreliable, inasmuch as some of these test animals now died before they gave evidence of the presence of frank tetanus. Bacteriological examination disclosed that the death of these guinea-pigs was due to a pasteurella type of infection and that the causative bacteria had been transferred to them in the blood of the sheep.

The amount of toxin that had entered the body of the recipient, *B*, was equivalent, according to our calculations, to 24.4 guinea-pig units per kilogram of its body weight—an amount that, if we may judge from the experiments summarized in Table III, probably represented as much as three minimal lethal doses. From the moment that the cross-circulation was started, one of these three lethal doses began to escape from the blood and to be fixed by the tissues of this animal in a non-recoverable manner; this fixation was completed in a very few hours thereafter, as may be judged from experiments with dogs described in Section VI. This permanently fixed fraction of the toxin sufficed to kill the animal by the 6th day after cross-circulation, and it would appear that the pasteurella infection did not hasten its death, inasmuch as the amount of toxin that was retained in its system during the cross-circulation was amply sufficient to cause death on the day it occurred.

The donor, Sheep *A*, with a much larger amount of toxin in its system, was found dead on the sixth day after inoculation, despite the fact that it must have contributed practically a third of its toxin supply to the recipient.

The following are among the interesting points that appear in connection with this cross-circulation experiment. It was seen that immediately following the interchange of blood between the two sheep, the toxin content of the blood of the donor dropped from 41 to 31 per cent, and then fell off during the next twenty-four hours to 24 per cent, after which it remained at the steady level of 24 to 27 per cent for the remaining two days of the life of the animal. The persistence of the toxin at this lower level for the three days following the cross-circulation shows that no appreciable amounts of it were liberated from organs or tissues that had fixed it, to pass back into the blood stream. The belief has often been expressed in the past that toxin that has been more or less firmly bound by the so-called non-reactive organs and tissues, may, under various circumstances, be freed from its union and again enter the blood stream.

Experiments of others on the toxin content of the blood of sheep infected with spores of clostridium tetani. As regards some earlier experiments on the presence of tetanus toxin in the blood of experimentally infected sheep, those of McClintock and Hutchings may be named. These authors were primarily interested in the possibly good therapeutic effects of various drugs, such as carbolic acid, ammonium sulphate, chlorbutanol, as well as tetanus antitoxin, on the course of the disease. They infected sheep by introducing splinters of wood infected with the spores of *clostridium tetani* under the skin of the tip of the tail. Thereupon the blood of these animals was tested daily for the presence of toxin by injecting 2 cc. of it into a hind leg of a single guinea-pig weighing 350 grams. The first symptoms of descending tetanus appeared in most of the infected sheep not earlier than the seventh day after infection. Their blood, however, from the third to the fourth day after the implantation of the infected splinters, contained enough of the toxin to kill guinea-pigs injected with 2 cc. of it; equal volumes of it, taken on other days and injected into guinea-pigs, produced tetanic symptoms but no deaths. While McClintock and Hutchings did not assay the amount of toxin circulating

in the blood with the desired degree of accuracy, as may be seen from their protocols, their findings nevertheless show that in sheep that had been experimentally infected with *tetanus spores*, the same conditions obtained in reference to the appearance of toxin in the blood during the period of incubation as in our experiments with *tetanus toxin*. We, however, were in the more advantageous position of being able to determine, in terms of guinea-pig units, not only the amount of toxin injected into our animals, but also that large part of it that remained in the blood and lymph and the smaller fatal part that was bound by the tissues in a manner that evaded detection. This very naturally could not be done by McClintock and Hutchings in their method of simulating the course of the natural disease. When the blood-toxin data of the experiments on sheep, to which these authors restricted themselves, incomplete as they are from a statistical point of view, are supplemented by our own bio-assays of the amount of toxin that is retained by both blood and lymph and the amount that is no longer detectable, we can make a probable estimate of the amount of toxin that must have been elaborated in the area into which the spore-bearing splinters had been introduced. On taking these factors into consideration, together with the body weight, blood volume, and the duration and intensity of the disease, we may be permitted to infer that, in most of the sheep studied by McClintock and Hutchings by their method of simulating natural tetanus, about 12 lethal doses of toxin must have been elaborated at the site of infection.

Toxin content of the blood of horses. As has been stated above, the sensitivity of the horse to tetanus toxin is thought to equal that of man; it is certainly so great that relatively small quantities of it are lethal for this animal. For this reason the results that were obtained in the bio-assays on the toxin content of the blood of Horse no. 1 and Horse no. 2 call for special study and consideration (Table III). Horse no. 1, a healthy mare, eleven years old, weighing 436 kilograms, was injected *intramuscularly* but superficially, into the shoulder with 0.5 cc. of toxin solution that contained 5 guinea-pig median lethal doses for every kilogram of the horse's weight; the quantity injected, therefore, amounted to 2,180 guinea-pig units. Within so short a time as five hours after the injection a considerable amount of toxin appeared in the blood of this animal, for the injection of 2 cc. of it

into guinea-pigs induced complete rigidity of their hind legs in four to five days without any signs of general tetanus. During the subsequent 24, 48 and 72 hours, the blood became more toxic, since 1 cc. of it now sufficed to stiffen the legs of the pigs, but even 3 cc. had no other effect than to induce local tetanus; from this time on its toxin content decreased slowly, so that after 96 and 120 hours, 2 cc. of it were again required to produce symptoms of local tetanus in the test pigs. On the 6th day (144 hours) after it had been inoculated, the horse was found to be *in extremis* and was therefore shot. There was so little toxin in the blood taken one-half hour after death that guinea-pigs inoculated with it in 3 cc. quantities showed no symptoms of tetanus.

The data on the quantities of the blood of this horse that were required to stiffen the legs of guinea-pigs, make it perfectly clear, that the toxin was slowly transferred from the site of injection into the blood stream, that it remained there at a constant level for three days, and that the horse gave no signs of its presence during these days. On the fourth and fifth days there was a slight decrease in the toxin content of the blood; the first symptoms of descending tetanus appeared on the fifth day. On the sixth day the animal lay prone, with extended legs, and was seized with such violent convulsive spasms that it seemed wise to shoot it.

The blood of Horse no. 2, that had received subcutaneously 4 guinea-pig units per kilogram of body weight, in place of the 5 units given to Horse no. 1, contained no demonstrable amount of toxin when tested on guinea-pigs in 6 cc. quantities. Six cc. portions of its *serum* (equal to 10 cc. blood) were also tested on the 6th, 7th, 8th, 12th and 13th days, respectively, but with entirely negative results. This horse was a 6-year-old mare, weighing 390 kilograms, and was in perfect health when injected with the toxin. The first noticeable symptom of tetanus, consisting of a slight difficulty in eating, appeared on the 8th day after the inoculation. On the 9th day there had developed marked symptoms of descending tetanus—pronounced trismus, the characteristic position of the ears and the nictitating membrane, arched tail, and slight stiffness in gait; on the 10th day, the picture was much the same except for an increased degree of trismus; on the 11th day, the trismus was complete, and

there was also profuse salivation; on the 12th day, the symptoms were much the same except for a more rapid respiration. At about 8 A.M. on the thirteenth day, the horse suddenly fell to the floor, and never thereafter regained its feet. As in the case of Horse no. 1 at this stage, convulsions of such extent and violence now appeared that the animal could not be approached without danger. The legs were perfectly rigid and were held spread apart, in the intervals between the seizures. The neck was rigidly arched, salivation was profuse and perspiration was so excessive that the animal's coat appeared to have been drenched with many buckets of water. As the convulsions decreased in intensity, the rectal temperature could be taken and was found to be 106.6°F. Respiration now became more and more inadequate, owing to the increasing rigidity of the essential and accessory muscles concerned with this function. The animal was shot three hours after it had fallen to the ground.

We have seen that Horse no. 2 suffered from a fatal tetanus of extreme degree following the subcutaneous injection of so small a quantity as 4 guinea-pig units per kilogram of its body weight. Its blood contained no demonstrable amount of toxin, since such massive doses as 6 cc. of its serum had no effect whatever on guinea-pigs. In contrast with this fact it was pointed out that the blood of Horse no. 1, which had received only one-fifth more of the toxin per unit of body weight, contained demonstrable, though not accurately assayed amounts of the toxin.

It is of interest to compare the above assays of the blood of our two horses, that had received well assayed fatal amounts of toxin, and those obtained by Permin with the blood of two horses that had died of accidentally acquired tetanus. The length of the incubation period for one of the two was unknown, but the injection into a guinea-pig of so large a quantity as 10 cc. of the serum of this horse induced merely local tetanus without further symptoms. The other horse developed symptoms of tetanus ten days after it had stepped on a nail, and 1 cc. of its serum likewise caused only local tetanus when injected into a mouse. We have here two fatal instances of the natural disease in horses, whose blood, like that of our Horse no. 1, that received 5 of our guinea-pig units per kilogram of its body weight,

contained only enough of the toxin to induce a local tetanic response but not death when injected into guinea-pigs or mice.

Permin also reports that he failed to find toxin in the blood of two of his patients, both of whom survived. One of the patients had rather severe tetanus, while the other showed a milder type of the disease. In neither case was the serum of the blood capable of inducing the slightest symptoms in mice, even when as much as 4 cc. was injected. The serum of the blood of two other individuals that died of severe tetanus was found to be highly toxic for mice, so much so that 1.5 cc. from one of the cases killed a mouse in two days. It has already been stated that many investigators were able to detect the toxin in the blood of human instances of tetanus, while perhaps an equal number were unable to obtain any evidence of its presence even when death occurred.

It appears to be well established, for many very susceptible animals as well as for man, that the toxin finds its way rapidly into the blood stream in increasing amounts as more and more of it is elaborated at the site of infection; and that none of it can be detected in the blood by our present methods in instances where the incubation period is of long duration and where just enough toxin is elaborated at the site of infection to lead ultimately to death. In case somewhat more of the toxin is elaborated than just suffices to kill, its presence may be detected in the blood, as is shown in our experiments, when known amounts of the toxin were injected, and by the observations of Permin on animals and human beings with the natural disease, and by those of other investigators that will be cited later.

Instances of excessive amounts of toxin in the blood of horses with natural tetanus. The literature of our subject contains some very striking examples of the truly enormous amount of toxin that can appear in the blood of horses with natural tetanus. An extraordinary, though perhaps not an unusual instance of this kind among the equidae, relates to a horse that was being immunized against diphtheria toxin by officials of the Board of Health of St. Louis in 1901. Large quantities of what was supposed to be uncontaminated antidiphtheritic serum were prepared from the blood drawn from this horse two days before it was known that the animal had become in-

fected with the *clostridium tetani*. This serum was used by physicians of St. Louis in the treatment of children during an epidemic of diphtheria, unfortunately with grave consequences.[10] Seven children, varying in age from four to eleven years (the ages being 4, 4, 4, 8, 8, 10, and 11) were injected with this serum in quantities varying from 5 cc. for the younger to 10 cc. for the older ones. In spite of the use of antitoxin all of these children died of tetanus. Four other children had severe tetanus but survived, and more deaths would undoubtedly have occurred had the serum been distributed over a wider territory.

Bolton, Fisch and Walden were the members of a committee appointed to look into the matter and to study the tetanogenic strength of samples of the serum that had led to the death of the children. Specimens of the serum that still remained in some of the vials used by the physicians were found to be extremely tetanogenic and lethal when injected into full grown guinea-pigs. Their data on test guinea-pigs are insufficient from a statistical point of view, but no doubt can be entertained in respect to the deadly action of the serum, since one-tenth of a cubic centimeter of it sufficed to kill a guinea-pig within five days.

Symptoms of tetanus developed in the horse two days after withdrawal of the tetanogenic and antidiphtheritic blood. The horse was described as being a vigorous animal, weighing 727 kilograms. We have calculated that the total volume of its serum amounted to 34,000 cc., and if we assume, as Bolton and his colleagues did, that the average killing dose of the serum for children of ten years of age or younger was 10 cc., the total amount of serum in this animal's body would have sufficed to kill 3,400 children of the assumed age and sensitivity to the toxin; and by calculation we also find that it would have killed 280 horses of a weight and sensitivity equal to that of the St. Louis horse. But this amount of toxin, large as it is, represents but one-half of the total amount of active toxin that was actually present in the body of the horse. We have shown that in sheep an equilibrium exists between the amount of toxin in the blood and that in the lymph, in that equal amounts of toxin are found in equal volumes of the two

[10] See also F. Siegert: Tetanus mit tödlichem Ausgang in Folge von Diphtherie-Heilserum-Injectionen in Italien. Münch. med. Wchnschr., xlviii, (I), 1901, p. 166.

fluids. Forty years ago Ransom showed that an equilibrium of this kind exists also in the much less susceptible dog, after the injection of varying amounts of toxin. By reason of this well established equilibrium, we should therefore have to double the amount of toxin that was found in the blood of the St. Louis horse in order to arrive at the sum total in its blood and lymph together. But quite aside from the calculations that are here presented, the incontrovertible fact remains that the serum of this horse contained in 0.1 cc. enough toxin to kill a full grown guinea-pig. No other instance is known to us of such a highly and almost unbelievably toxic blood as was found in this animal with natural tetanus.

A somewhat similar case was reported by Madsen of a horse that was being immunized in his Institute in Copenhagen, but in this instance the serum contained materially less tetanus toxin than that of the St. Louis horse. Blood was drawn by Madsen on March 21, 1908, and the serum was found to be lethal for guinea-pigs in amounts of 5 to 10 cc., injected subcutaneously. The horse showed no symptoms until March 26, when it died within 12 hours after the initial manifestations of the disease. In this instance, however, the serum had not been sent out to physicians and therefore no fatalities occurred. Madsen calculated that at the time the serum of this horse was assayed its blood contained an amount of toxin that would have sufficed to kill three other horses.

Bolton and Fisch, after making their examinations of the tetanogenic antidiphtheritic serum of the St. Louis horse, made a number of studies on the content of tetanus toxin in the blood of horses that had been experimentally infected by means of subcutaneous implantations of garden earth. Their findings are analogous to those reported by McClintock and Hutchings for sheep that were experimentally infected with tetanus spores.

IV. FURTHER EXPERIMENTS BY OURSELVES AND OTHERS IN REFERENCE TO THE ABSORPTION AND DISPOSAL OF THE TOXIN BY THE BLOOD, LYMPH AND ORGANS OF SOME OF THE DOMESTIC ANIMALS

Experiments on dogs. Ransom, working in Behring's institute at Marburg thirty-five years ago, alone, among earlier investigators, attacked in a serious manner the problem of the distribution of both

tetanus toxin and antitoxin between the blood and lymph. This investigator experimented with dogs and assayed both the injected toxin and that found later in their blood and lymph, by the use of white mice, in accordance with the method of Behring. He showed that toxin injected into the blood, passes so freely into the lymph, that the two fluids contain equal amounts of it in equal volumes, after the lapse of 26 hours. While the data obtained by Ransom are subject to criticism from the statistical point of view, they are nevertheless of great value. After the injection of a known amount of toxin into the blood stream (1.4 to 1.6 minimum lethal doses for the dog), it was found that about 26 per cent of it had disappeared from both the blood and the lymph in 3 hours, while after the lapse of 26½ hours, more than 70 per cent of the injected toxin had disappeared from the two fluids; after 33 hours the blood and lymph together contained only about 3 per cent of the originally injected toxin. Ransom's experiments indicate that there is a marked difference in the ability of the blood-lymph vascular system of the dog, as contrasted with that established by us for the sheep, to retain toxin that has been taken up by it. While this system of the dog loses more than 70 per cent in the course of 26½ hours and 97 per cent in the course of 33 hours, the exact reverse holds true for sheep. In their case the blood and lymph together continue to retain more than 90 per cent of the originally injected toxin throughout the days intervening between the time of the injection and that of death.

Our experiments with dogs. We have summarized in Table V the results of a number of experiments in which the toxin-content of the blood of dogs was determined after intravenous administration of varying doses of the poison. While the number of experiments is not large, we would state that the percentages of recovered toxin there recorded have been repeatedly corroborated by the blood-toxin assays recorded in Section VI in connection with the determination of the length of time required for the fixation by the body of a lethal dose of the toxin.

The bio-assays made in connection with Dog X-4 showed that when a harmless dose of toxin (120 G.P. units per kg.) is injected intravenously, the toxin is retained in very considerable amounts during the first 48 hours after injection, and that a trace of it is still present after

TABLE V

*Showing the percentage of tetanus toxin in the blood of dogs at various intervals after intravenous injection of varying doses**

DATE	DOG	WEIGHT	DOSE PER KILOGRAM IN L.D. 50's	PERCENTAGE OF INJECTED TOXIN IN BLOOD AFTER							
				10 min.	1 hr.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	
1936		*kgm.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
May 4	X-4	11.3	120	52	52	25	17 approx.	Trace†	Trace	Not tested	Dog remained entirely normal; no symptoms of tetanus appeared during 3 weeks of observation
May 4	X-5	11.3	240	41	55	20	13	8	Trace	Not tested	Dog developed typical tetanus; died on 14th day
May 21	X-9	9.5	1,406	Not tested	Not tested	6 approx.	Less than 4	Less than 2	Trace	Not tested	Dog, not confined to cage, developed typical tetanus in the extremities on 4th day; etherized to death
June 2	X-11	6.9	1,406	Not tested	Not tested	8 approx.	Less than 4	Less than 1	Trace	0‡	Dog placed in a cage after the injection and kept there until death. Developed typical tetanus and died on 8th day. Onset of symptoms and death delayed by restriction of activity. (See Papers I and III)

* The assays were made by injecting the serum into guinea-pigs.

† Trace indicates that 3–6 cc. of serum caused stiffness but not death in guinea-pigs in 5 days.

‡ 0 indicates that 6–8 cc. of serum produced no symptoms in guinea-pigs.

96 hours. The bio-assays of the toxiferous blood of a dog (X-7, not entered in the table), that had received the same quantity of toxin as Dog X-4, not intravenously but subcutaneously into the left mid-thigh, showed (in the earliest test made) that 8 per cent of it was present in the blood 24 hours after injection, while after 72 hours none of it could be detected, inasmuch as so large a quantity as 8 cc. of serum failed to elicit any symptoms when injected at this time into guinea-pigs.

Dog X-5 received intravenously twice as much toxin as Dog X-4 (namely 240 G.P. units per kg.). This amount of toxin induced a slowly developing tetanus that caused death on the 14th day; this quantity approximates, though not very closely, the median lethal dose for dogs when the interval between injection and death is limited to six or seven days. It will be seen that the percental figures for the blood-toxin content of Dog X-5, that died of tetanus, were much like those given for Dog X-4 (that remained unaffected by half the dose that killed X-5), except that the toxin persisted in its blood in measurable quantities for a longer time, inasmuch as 8 per cent was still present 72 hours after injection, whereas only a trace of it could be found in the blood of X-4 at this time. A few bio-assays of the blood of a dog (X-6, not given in the table), that had received subcutaneously the same amount of toxin per kilogram as the intravenously injected Dog X-5, showed that its blood, which was not assayed until 24 hours had elapsed, contained 15 per cent of the toxin—an amount that differs but little from that found at this time in the intravenously injected animal. After 48 hours and after 72 hours, only a trace of the toxin could be found in the subcutaneously injected animal. The intravenously injected Dog X-5 died from a severe and typical tetanus on the 14th day; the subcutaneously injected one, X-6, developed typical tetanic symptoms with marked stiffness of the muscles of the entire body, but finally recovered. The two series of experiments seem to show that when equal quantities of toxin are injected intravenously or subcutaneously into dogs, the blood retains the toxin in greater amounts and for a longer time in the former case than in the latter. We surmise, however, that this difference will not obtain for very much larger doses than were here employed.

We have given the above data on the toxin-content of the blood of

the dog after intravenous or subcutaneous injections of amounts of the toxin that approach the median semilethal and the median lethal dose, because other investigators, as far as we can recall, have not assayed the blood of this animal for its toxin-content after such small doses, more especially when they were injected beneath the skin. We were not a little surprised to find that the injection of such doses of the toxin by this route, as in Dogs X-7 and X-6 (not given in the table), led to the appearance, respectively, of 8 and 15 per cent of the injected toxin 24 hours later. The blood of guinea-pigs, of the horse or of the sheep, holds no such proportionate amount of their semilethal or median lethal dose (given subcutaneously) as does that of the relatively much less susceptible dog. We have here another instance of the statement emphasized by us elsewhere in this paper, that there is no uniformity in the manner in which the circulation of different species of animals responds to the injection of a fractional or of an entire lethal dose of the toxin.

The bio-assays of the toxiferous blood of Dogs X-9 and X-11, each of which received intravenously six times the quantity of toxin given to Dog X-5 (namely, 1,406 G.P. units per kilogram of body weight), stand in good agreement with those obtained by Ransom, who injected fatal quantities of toxin that were somewhat smaller than those employed by us. It is not easy to determine how much more we injected than he did, since he employed the Behring mouse unit for the standardization of his toxin and for his blood assays, while we used the guinea-pig median lethal dose for our standard. As stated above, Ransom found that 33 hours after injection the blood and lymph together contained only about 3 per cent of the injected toxin. We made no tests at the 33-hour period, but our table shows that after 48 hours the blood alone of Dogs X-9 and X-11 contained less than 4 per cent of the injected toxin. Our findings and those of Ransom on the toxin content of the blood after intravenous injection of several lethal doses are not entirely in agreement but the difference is nevertheless negligible, in spite of the fact that we made use of the sensitive guinea-pig as our test animal and estimated the toxin content of the blood in conformity with statistical methods.

Toxin in the cerebrospinal fluid. In order to learn whether toxin escapes from the blood into the cerebrospinal fluid, Ransom injected

into the jugular vein of a dog approximately six minimal lethal doses of the toxin (according to our calculations). Twenty-four hours later, the cerebrospinal fluid contained only the merest trace of the toxin, but at this time one-fifteenth of the originally injected quantity was still present in the blood. We examined the spinal fluid of only one of our sheep (no. 6) and found it devoid of toxin at a time when the toxin-content of the blood was at its maximum. Our assays of the fluid withdrawn from the cisterna magna of the rabbit also gave no evidence of toxin in two instances in which it was tested on 10-gram mice, in quantities of 0.3 cc. One rabbit received 2 minimal lethal doses intramuscularly, the other an equal amount intravenously, and the cisternal liquor was withdrawn and tested 48 hours later.

Stintzing (1898) was the first to make bio-assays of the cerebrospinal fluid of a human being with tetanus. Under light anesthesia, he removed 6 cc. of the perfectly clear fluid, by lumbar puncture, from a vigorous workman, on the 14th day after the man had sustained an injury of the groin, and found it to be very toxic for mice. Unfortunately, the blood was not tested at this time. On the 16th day, less than 24 hours before death occurred, the spinal fluid was found to be much less toxic than before, while the blood, which was now assayed for the first time, was stated to be free from it.

Permin reports that he found toxin in the cerebrospinal fluid of three of his patients with tetanus, only one of whom recovered—a lad of seven years, who showed the first symptoms of tetanus eleven days after a thorn had penetrated his foot. Thirty hours after the first signs of tetanus, the lumbar spinal fluid of the lad is stated to have killed two mice, one of which had received 1 cc. and the other 3 cc. Not the slightest evidence, however, was offered to support the conclusion that the two mice actually died of tetanus. The case was one of very mild tetanus, ending in recovery, and we cannot admit that Permin really proved that toxin was present in the spinal fluid of this case, especially in view of the fact that he described in detail the manner in which mice reacted with true symptoms of tetanus to the spinal fluid taken from his other instances of a severe and fatal tetanus, in one of which the blood was actually tested and found to contain more toxin than was present in the lumbar fluid.

We cannot here consider the many other instances of human tetanus

in which the spinal fluid was tested, generally with negative results, but it cannot be doubted that the toxin appears in the cerebrospinal fluid in some severe forms of the disease at a time when the blood is laden with toxin.

Rabbits. According to Knorr (1895), the following conditions hold for the rabbit as regards the retention of toxin by its blood, subsequent to intravenous or subcutaneous injection. This observer found that the serum of the rabbit contains no demonstrable toxin in the 24 hours following the subcutaneous injection of a *single* minimal lethal dose, or at any time thereafter; following the subcutaneous injection of 10 such doses, however, it was found that a small fraction (1/200) of the injected toxin could be detected in the blood or serum after three hours, and somewhat more of it after twenty-four hours, and that it persisted there until death occurred.

Marie, whose observations confirmed those of Knorr, found that on injecting a rabbit *per venam* with 10 cc. of a diluted toxin of such strength that it sufficed to kill 100,000 mice (approximately 10 minimum lethal doses?), 1 cc. of its defibrinated arterial blood, taken 17 hours after the injection, killed mice within three days; 1 cc. of the defibrinated blood, taken later, had but little effect on mice, and it was therefore assumed that the blood now contained only mere traces of the toxin. Marie is in agreement with Knorr in believing that when toxin is injected subcutaneously into rabbits it can be detected in their carotid blood for a longer time and in larger quantities than when it is injected by the intravenous route. This supposition is based, however, on insufficient evidence, and is, furthermore, not in agreement with certain data presented by Knorr himself in one of his tables (Cf. his Table VIII).

The results of Wolff-Eisner's experiments on the toxin content of the blood of rabbits, that had received intravenously amounts of toxin varying from 0.01 to 0.1 gm. of the dry preparation, cannot be reconciled with those obtained by Knorr and Marie and those of other authors, still to be cited. On injecting 0.01 to 0.03 gm. of his toxin and testing the rabbit's serum three hours later on mice, he found it to be non-toxic; when he injected 0.05 gm. of his toxin—an amount that killed the rabbit in so short a time as 34 hours—the serum was found to be only slightly toxic for mice. These results have been cited

occasionally as proving that even large doses of toxin, when injected intravenously into rabbits, disappear from the blood stream in the short interval of three hours. Wolff-Eisner's failure to detect the toxin in the blood after the injection of large doses is to be ascribed to the fact that he limited himself to the injection of so small a volume of serum as 0.25 cc. into mice that weighed from 15 to 20 grams. Had he injected larger quantities of it into mice and had he also tested it on the more sensitive guinea-pig, his findings, we believe, would not have been so entirely contrary to those of all other experimenters in the field. Aside from the fact that Wolff-Eisner employed only minimal quantities of serum, his work is also very defective from a statistical point of view.

The investigations of Knorr and Marie were preceded by the less frequently cited ones of Kartulis which were carried out in 1892 in Koch's laboratory in Berlin. This author corroborated some earlier observations of others in regard to the toxin content of the blood of human beings with tetanus, as also of that of animals injected with the toxin.

Toxin in the urine. Kartulis also found that the toxin is excreted by the kidneys of rabbits and guinea-pigs only after these animals had been inoculated with excessive amounts of toxic filtrates, but not when they were infected with splinters of wood impregnated with the *clostridium tetani* and its spores. That the toxin failed to appear in the urine, when the animals were inoculated with spores, can be due only to the fact that the amount elaborated by the bacilli at the site of infection did not now suffice to attain the high threshold level in the blood requisite for urinary secretion. These observations in respect to the appearance of the toxin in the urine of experimental animals, like the earlier ones of Bruschettini and Brunner and those of Bieling and Gottschalk (1923), show that the threshold for the urinary excretion of the toxin is surmounted only when *excessively large quantities of the toxin* are present in the blood.[11] Evidence in favor of this statement is found in the fact that Kartulis failed to find toxin in the urine of six patients that succumbed to severe tetanus. His negative findings with the urine of human cases of tetanus cor-

[11] For further information in regard to the appearance of tetanus toxin in the urine of experimental animals and of human beings with tetanus, see a critical and controversial paper by Brunner: Ztschr. f. klin. Med., 1897, xxxii, 207.

roborated those of Brieger, Nissen and Stern, and other instances of the kind are also reported by Blumenthal, Jacob and others. The failure to find toxin in the urine of so many cases of human tetanus has served to modify the earlier erroneous belief of Bruschettini, who, on finding the toxin in the urine of two patients with a fatal tetanus, concluded that it is generally excreted by the kidneys and that urinary tests for it would therefore be of great service in establishing a diagnosis of tetanus. There can be no doubt that toxin may appear in the urine of human beings when a great excess of it is being elaborated at the site of the infection.

We may now return to a consideration of the experiments of Kartulis on the time during which the blood harbored the toxin after the *subcutaneous* injection of quantities of his toxic filtrate that varied from 50 to 5 cc. According to our calculations, which are based on such imperfect data as were given by him, these quantities of filtrate contained, respectively, 25 to 2½ minimum lethal doses for his unweighed rabbits, which we have assumed to weigh 2 kilograms. We assume also that his filtrates were assayed on mice weighing 15 grams, as here also no weights were given. Making use of the assumed weights for his rabbits and mice, the experimental data of Kartulis work out as follows: In Experiment *A* (page 11 of his Dissertation) a rabbit was injected subcutaneously with 50 cc. of a toxic filtrate equivalent to 25 minimal lethal doses of the toxin, and thirty hours later, when the animal was in severe tetanus, it was bled to death and 0.1 cc. of its serum was then found to be fatal for a mouse within four days, while 0.3 cc. of it killed a mouse in 30 hours. At the time this rabbit was killed, which could not have been very many hours before it would have succumbed to tetanus as a result of the 25 lethal doses of toxin, its blood content, according to our calculations, was approximately 1.6 per cent of the toxin that had been injected into it. On making a similar calculation (leaving out of consideration Rabbits *B* and *C*) for an experiment with Rabbit *D*, that had received subcutaneously only 5 cc. of the toxic filtrate—the equivalent of 2½ lethal doses of toxin—and whose serum was assayed 75 hours later, we find that only 0.27 per cent of the toxin was present in the serum at this time. These results are not far out of line with those obtained by Marie and Knorr, as given above.

It is quite apparent from the work of Kartulis that the injection of

increasingly large amounts of the toxin into rabbits and guinea-pigs is followed by the appearance of correspondingly large amounts in their blood. The ability of the rabbit's tissues to dispose of a certain excess of toxin, over their already large lethal dose, is so marked that only a small part of this excess is retained in the blood stream for two or three days. At what point, however, this ability of the rabbit to dispose of the toxin fails to keep pace with the injection of really excessive amounts, as, say 600 or more lethal doses (amounts that have frequently been injected into guinea-pigs), we are not informed, since no one has assayed both the blood and the lymph of this animal after the injection of such massive doses.

Blumenthal (1898), to be sure, gives an account of numerous experiments with rabbits that were injected subcutaneously with 100 to 200 lethal doses (?) and killed at intervals of 17 to 20 hours and 24 to 30 hours thereafter; at these hours the blood and various organs were assayed for toxin. He concludes a brief and categorical account of his experiments with the italicized statement, that "never, at the beginning of tetanus (at 40 hours), was there a trace of toxin to be found in the body of the rabbits." Inasmuch as Blumenthal's rabbits did not show the first symptoms of tetanus until 40 hours after the subcutaneous injection of the postulated 100 to 200 lethal doses, we may be quite certain that he did not employ such excessive quantities. The literature of tetanus contains many similar instances of a faulty bio-assay of what actually constitutes a lethal dose for a given animal.

Teale and Embleton (1919) showed definitely that when rabbits are injected subcutaneously with so large an amount as 10 cc. of a potent toxin (number of lethal doses not stated), it appears in the chyle in eight hours to such an extent that 0.5 cc. of this fluid killed a mouse in three days; the authors also made the important observation that in so short a time as 12 hours an equilibrium is established between the amount of toxin found in the serum and that found in the lymph, in the sense that equal volumes of the two fluids kill mice in approximately the same time; after 48 hours, at a time when the rabbits showed marked symptoms of tetanus, these fluids "no longer contained enough toxin to give rise to even local tetanus" when injected into mice in the quantities of 1 to 0.5 cc. The authors add (Experiment 4): "The lymphatic glands also had very little. The

same was the case with the spleen. The other viscera apparently contained no toxin."

The interesting fact emerges from the experiments of Knorr, Marie, Kartulis and Teale and Embleton, that tetanus toxin disappears almost completely from the blood and lymph and the various organs of the rabbit, in the course of two or three days, after this animal has been inoculated with 10 to 20 lethal doses. The few experiments that we have incidentally made support this conclusion. It must not be thought, however, that the blood and lymph of the rabbit may not, in fact, retain a relatively very large percentage of the toxin when several hundred lethal doses of it come into consideration. It is to be noted in passing that the disappearance of the toxin from the blood takes place during the incubation period, at a time, therefore, before any tetanic symptoms have made their appearance.

The rapid disappearance of large quantities of toxin from the blood of the rabbit and the dog, after the injection of a certain number of lethal doses, is no doubt responsible for the onesided opinion, so definitely stated in modern hand-books and text-books on bacteriology that "toxin does not appear to be absorbed by the blood to any great extent, since the toxic content of the blood is low." Our findings, as given in Section III, of the high toxin content of the blood of sheep, and those reported by others for horses and human beings with natural tetanus, show that a generalization of this character is not justifiable.

Guinea-pigs. It has long been known that the guinea-pig must be classed with animals that are highly susceptible to tetanus toxin, such as the horse, the sheep, the monkey and the human being. In tables recording the relative amounts of toxin required to kill 1 gram of animal, the guinea-pig is placed next to the horse, and its usefulness in bio-assays of the toxin is based on the fact that in an animal of its small size and high susceptibility symptoms of tetanus can be brought to light by injecting it with very small doses of the toxin. A few investigators have attempted to show that the body of this animal, whose voluntary muscles and central nervous system can be fatally poisoned by a very minute dose of the toxin—an action that has placed it in the class of the most highly susceptible animals—is also endowed with the ability to eliminate very rapidly from its blood many multiples of a fatal dose.

Immediately after Behring and Kitasato had shown, in 1890, that the blood of the relatively unsusceptible rabbit can be made to acquire antitetanic properties by an appropriate treatment of the animal with the toxin, Pestana (1891) undertook some experiments with the object of studying the relative distribution of the toxin among the organs of the guinea-pig. His results were given in a short paper of two pages, but with such lack of details and of statistical evidence that their value is of a limited and purely qualitative character. He was content to show that after the *subcutaneous* injection of the toxin, into guinea-pigs, it finds its way into the blood, but he drew no deductions in reference to the percentage of toxin in the blood in terms of the amount originally injected; to do so would have required much more careful and detailed work on his part.

A few years after Pestana, Quadu, working in the laboratory of Vincenzi, also made some experiments in respect to the appearance of the toxin in the blood of guinea-pigs subsequent to the injection of varying amounts of it. This author made use of adult guinea-pigs weighing 600 grams and determined what he called the minimal lethal dose of the toxin for guinea-pigs of this weight that received it by subcutaneous injection. Small guinea-pigs, weighing 300 grams, were used as test animals for the blood in place of white mice. The 600-gram pigs were injected with a *single*, previously determined, lethal dose of the toxin and were then bled to death at various intervals thereafter. The volume of the collected blood amounted to 20 cc.; at times this entire amount of the blood was injected subcutaneously into the smaller test guinea-pigs, at other times, the serum only; in neither case did the test animals ever respond with any symptoms of tetanus, not even with local tetanus—a symptom that is always brought out by very minute doses of a toxin. Quadu next injected the large guinea-pigs intravenously with a quantity of toxin equivalent to six times the subcutaneously determined minimal lethal dose, and tested the blood at various times. He now noted the appearance of tetanic symptoms in his test animals, but leaves us entirely in doubt in regard to their course and severity. It is quite impossible, in view of the indefinite character of Quadu's experiments, to arrive at any conclusion in respect to the percentage of toxin in the blood of his 600-gram guinea-pigs in terms of the injected amount. Quadu's

results are of significance for the problem under consideration only as showing that the blood of his large guinea-pigs that had received 1 minimal lethal dose did not contain enough of the toxin to cause the slightest symptoms of tetanus when injected into the smaller test pigs.

A few years ago, Yasuyama and Schöbl also made some approximately quantitative determinations of the amount of toxin present in the blood of inoculated guinea-pigs; they undertook this task after observing that 5 cc. of the venous blood of an eleven-year-old boy with fatal tetanus, injected into a guinea-pig, was followed by symptoms of tetanus on the second day, and by the death of the animal on the fourth day. These authors thus added another to the many instances on record in which the blood of human beings with tetanus has been shown to harbor considerable amounts of the toxin. In the course of their experiments, Yasuyama and Schöbl injected guinea-pigs of standard weight with quantities of the toxin that varied from 2 to 600 minimal lethal doses and tested their venous blood on mice of standard weight and guinea-pigs weighing 300 grams. We can give here only two examples of their findings. It was found that when 200 such lethal doses were injected into a guinea-pig weighing 300 grams, 0.5 cc. of its venous blood, withdrawn 24 hours later and injected immediately into a normal guinea-pig of the same weight, killed it within three days. Assuming that the total blood-volume of the donor guinea-pig amounted to 20 cc., it is apparent that its blood harbored, after twenty-four hours, enough of the toxin to kill 40 normal guinea-pigs of the same weight, and that therefore 25 per cent of the 200 lethal doses originally injected was still present in the blood 24 hours later, while an equal amount of the toxin was certainly present in the lymph at this time.

When 60 minimal lethal doses were injected into a guinea-pig it was found that 0.05 cc. of its venous blood, taken 24 hours later, killed a mouse weighing 15 grams in three days. When allowance is made for the relative susceptibility of guinea-pigs and mice to the toxin, it may be concluded that the blood of the guinea-pig that had received the 60 minimal lethal doses harbored, after 24 hours, about 20 per cent of the injected toxin. When the guinea-pigs received only 20 lethal doses the toxicity of their blood was correspondingly lower,

since 0.5 cc. of it now enabled a 15-gram mouse to survive until the sixth day after injection. After 2 minimal lethal doses, the blood of the pigs, taken 24 hours later, was found to be non-toxic for mice in doses of 0.25 cc. This last observation confirms our own belief and that of earlier writers that the blood of guinea-pigs inoculated with a minimal lethal dose of the toxin retains so little of it that its presence can be detected only with difficulty, if at all, by injection into mice. In this respect, then, the guinea-pig must be classed with the other highly susceptible animals, such as the horse and man, whose blood likewise harbors, after a single lethal dose, too small an amount of the toxin to be assayed by means of the test animals now employed.

Yasuyama and Schöbl, as we have seen, found that guinea-pigs that had been subcutaneously injected with such excessive amounts of toxin as 200 to 600 lethal doses still retained from 20 to 25 per cent of it in their blood at a time when death was imminent. To this we must add the 20 to 25 per cent of the toxin present in the lymphatic system. Equilibrium in respect to the toxin content of the blood and lymph of the guinea-pig is probably established in less than ten hours, in view of the fact that it is effected in twelve hours in the rabbit while somewhat more time is required in the case of larger animals, such as the dog and sheep. Inasmuch as Yasuyama and Schöbl made their blood assays 24 hours after injecting the toxin, it is safe to say that the blood and lymph of their guinea-pigs, taken together contained approximately 50 per cent of the 200 to 600 lethal doses with which they had been inoculated, and this at a time when death was certainly imminent; we find that guinea-pigs that have received intravenously only 100 lethal doses die in 24 hours or less.

There is every reason to believe that the disappearance of the toxin from the blood is accelerated, in the last hours of life, not only in the guinea-pig but also in the sheep and other animals, in consequence of the profound metabolic disturbances that have appeared by this time.

Bieling and Gottschalk (1929) made a number of experiments to determine the toxin content of the blood of guinea-pigs, the extent to which the toxin is absorbed and retained by the spleen, kidneys, liver, muscles and brain, and under what circumstances it appears in the urine. They injected directly into the heart of the guinea-pigs, 100 to 400 lethal doses, and claimed to have found that the voluntary

muscles and the viscera, but more particularly the spleen, removed from the blood stream considerable quantities of the injected toxin even in the first thirty minutes following the injection, as shown by the data of the two experiments given in their Tables V and VI (pages 133 and 134). The amount of toxin injected into the heart was equivalent to what would have killed ten thousand mice in 1 to 2 days. It is stated in Table V that the serum in this half-hour experiment still contained so much toxin that $\frac{1}{50}$ cc. killed a mouse in 4 days. The ground up and washed organs were effective in the following dilutions: Spleen diluted 1 to 5 killed in 5 days, diluted 1 to 10, it was fatal in 4 days; liver diluted 1 to 2 fatal in 4 days; brain diluted 1 to 2 was without effect; kidney diluted 1 to 2 was fatal in 4 days, diluted 1 to 5 also fatal in 4 days; muscle diluted 1 to 2 was fatal in 5 days. The total volume of tissue extracts injected into test mice was 0.5 cc. When the sum total of the weights of all of the viscera and of the voluntary muscles of the guinea-pig weighing 320 grams used in this experiment is taken into account, the tissues according to these authors must have absorbed and retained a considerable amount of the injected toxin in the short space of thirty minutes. The exact point at which the diluted serum would have killed mice in two days was not given. One cannot do more than obtain a rough idea of the amount of toxin that was thought to have been lost from the serum in thirty minutes.

We may be pardoned for entertaining the belief that, in their experiments with the organs of two guinea-pigs, as given in their Tables V and VI, our authors did not completely remove the toxin that was still retained in the two great reservoirs of the blood and lymph systems after the intracardiac injection of several hundred lethal doses. The animals, to be sure, were bled to death at the end of thirty minutes, but this operation removed at the most not more than one-half of the toxin that was still present in the blood at the time.

Of more interest, however, is an experiment on a third guinea-pig, weighing 360 grams, that had received by intracardiac injection one thousand mouse units of the toxin and was not bled until five hours after the injection. Our translation into English of their findings in this connection is as follows: "While in the previous twenty-minute experiment [thirty minutes?] the serum was so richly laden with toxin

as to cause fatal convulsive tetanus in mice in 4 days, even in a dilution of 1–80, it now, in a dilution as low as 1–5, contained only a sublethal amount of toxin. Still more marked now is the disappearance (Rückgang) of toxin from the organs. None of the organs, even when tested in the highest concentrations at which it was technically possible to employ them, could any longer induce demonstrable signs of tetanus; even the spleen, which in the former [half-hour] experiments was so powerfully active, was now innocuous in a dilution of 1–4." We offer no criticism in respect to the fact that the emulsified organs were found to be free from toxin in the five-hour experiment, as this may be taken for granted in the case of all organs that have been properly washed, and this seems to have been the case in this particular experiment. Our concern here is rather with the fact that the blood serum is stated to have contained at this time only a minimal amount of the toxin, inasmuch as the injection of $\frac{1}{8}$ cc. of it into test mice caused only symptoms of a local tetanus. This would imply, as a simple calculation shows, that all but two per cent of the injected toxin had disappeared from the blood. But quite aside from this calculation, the conclusion at which Bieling and Gottschalk have arrived, as quoted above and as again stated by them in the summary of their second paper, to the effect that when the toxin is injected intravenously into guinea-pigs in such large amounts as 100 to 400 lethal doses, it disappears almost entirely from the blood stream in the brief period of five hours, is shown to be entirely erroneous by the concordant experiments of other writers, inclusive of those made by us.

It will be seen from our own bio-assays that five hours after the intracardiac injection of 100 to 400 lethal doses of toxin (the quantity employed by Bieling and Gottschalk), the blood of the guinea-pig still harbors 50 per cent of the injected amount. Bieling and Gottschalk, as we have seen, obtained negative biological tests with the well washed organs of guinea-pigs five hours after the injection of the stated amounts of toxin, at a time when they mistakenly believed the blood to be practically free from it; in reality, however, as is proved by our experiments, the blood harbored at that time 50 per cent of the originally injected toxin.

It may be added that account is taken by Bieling and Gottschalk of the fact that a certain amount of toxin is excreted in its native

form by the kidneys, but, inasmuch as this is the only route by which the toxin can be shown to escape from the body, the urinary loss is not of great importance. The authors conclude, that the great amount of toxin that was originally injected and that could no longer be found in the blood or in any of the organs "must therefore have been destroyed in the body or, in other words, have been rendered inactive." The statement as here made without qualification is also untenable, as will be pointed out in Section VI, where it will be made clear that we are not justified in believing that the toxin is always "destroyed or rendered inactive" in organs that have fixed it in a way that makes it undetectable by means of the biological tests now employed. The voluntary muscles and parts of the central nervous system are examples of organs that fix the toxin in a non-detectable manner, but which are nevertheless tetanized by it—a fact that is not reconcilable with the belief that a negative biological test is always indicative of destruction of the toxin.

The unsupported conclusions of Bieling and Gottschalk in respect to the very rapid disappearance of the toxin from the blood stream and its rapid "destruction" in the tissues of the guinea-pig have been widely quoted and are in part responsible for the unqualified statements previously cited, that the toxin does not appear to be absorbed or retained by the blood to any great extent, and that the toxin content of the blood is very low.

Our experiments with guinea-pigs. We may now proceed to give our own experiments, which deal solely with the toxin content of the blood of guinea-pigs subsequent to the administration of the toxin in the various doses employed by our predecessors. In carrying out our experiments, the results of which are summarized in Table VI, we assayed our toxic filtrates and the toxiferous blood of the injected guinea-pigs in accordance with the statistical principles described in Section II. Guinea-pigs, weighing approximately 350 grams, received known amounts of the toxin *per cor* or *per venam*, and, as noted in the table, their toxiferous blood was injected at certain intervals into normal guinea-pigs and mice of known weight. The details of the various assays need not be given, but it may be remarked that a sufficient number of guinea-pigs and mice were employed, in each series of tests, to determine which of them received just enough of the

TABLE VI

Showing the percentage of tetanus toxin circulating in the blood of guinea-pigs at various intervals following the injection of 100 and 400 lethal doses

DATE	GUINEA-PIG NUMBER	WEIGHT	ROUTE OF INJECTION	NUMBER OF LETHAL DOSES	TIME OF WITHDRAWAL OF TOXIFEROUS BLOOD AND AMOUNTS OF IT THAT KILLED TEST ANIMALS IN 4 TO 5 DAYS								CALCULATED PERCENTAGE OF TOXIN IN BLOOD AT VARIOUS INTERVALS AFTER INJECTION OF THE TOXIN					
					Test guinea-pigs of 350 grams					Test mice of 10 grams								
					30 min.	5 hrs.	17 hrs.	21 hrs.	24 hrs. after death	17 hrs.	19 hrs.	24 hrs. after death	30 min.	5 hrs.	17 hrs.	19 hrs.	21 hrs.	24 hrs. after death
1936		*grams*			*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*		*per cent*
Feb. 15	1	350	Intrajugular	100		0.4								50				
Feb. 20	2	325	Intracardial	100			0.75	2.0*				0.2			26			20
Feb. 26	3	350	Intrajugular	100			1.0		1.0	0.2	0.2	0.2			20	20		20
Mar. 17	7a	350	Intrajugular	100			1.0	2.0*		0.2					20			
Mar. 19	9	360	Intracardial	100		0.4	2.0*	2.0*						50				
Mar. 19	10	365	Intracardial	400		0.1								50				
Feb. 20	4	320	Subcutaneous	100			1.0	2.0*				0.3			20			13
Feb. 20	2a	320	Subcutaneous	100			2.0*	2.0*				0.3						13
May 5	1a	350	Intrajugular	100	0.2								100					
May 5	2b	350	Intrajugular	100	0.3								66					

* Smaller quantities not tested. Death occurred in the test animals in less than 4 days.

toxiferous blood to kill them in four to five days; this time limit for death meets the requirements of our statistical method, and details in respect to other test animals that died earlier or later are omitted from the table, in the interest of brevity.

A study of our bio-assays, as given in the table, shows that (1) heart blood withdrawn thirty minutes after intracardiac injection of 100 lethal doses is so richly laden at this time with the toxin, that so small a quantity as 0.2 to 0.3 cc. suffices to kill normal guinea-pigs in four to five days; (2) such blood drawn from the heart five hours after the intracardiac or intravenous injection of 100 to 400 lethal doses still harbors, after this interval of time, at least 50 per cent of the originally injected toxin. Seventeen hours after the injection of such large quantities of toxin, only 20 per cent of it could be recovered from the blood. At this time, the guinea-pigs are very near death, which regularly occurs in 18 to 20 hours after the intravenous injection of 100 to 400 lethal doses. In considering the results of the five- and seventeen-hour experiments it must be borne in mind that during these hours the blood has been constantly losing toxin to the lymphatic system, in accordance with explanations previously given.

V. ANIMALS VERY REFRACTORY TO TETANUS

It was proved nearly forty years ago that many of the lower vertebrata show an almost unlimited resistance to tetanus toxin[12] and that their blood stream serves as a storage place in which it may be retained for weeks and months and from which it ultimately disappears without causing symptoms either of a local or general tetanus. The marsh turtle, *Emys orbicularis*, affords an example of this class. When the toxin is injected into this animal, subcutaneously or intraperitoneally, it passes quickly into the blood, which may still be tetanogenic four months after the injection. It is stated that in the grown alligator, which is also very refractory to tetanus, the toxin is eliminated from the blood stream more rapidly than in the turtle, when it is kept at the relatively low temperature of 20°C. At this temperature the blood does not become antitoxic, although it has lost its tetanogenic property. Antitoxin is, however, produced, often with great rapidity,

[12] Metchnikoff, E.: Immunity in Infective Diseases. Translated from the French by Francis G. Binnie, Cambridge University Press, 1905.

when grown alligators are kept at the higher temperatures of 32° to 37°C. The high immunity of the alligator to tetanus cannot be accounted for on the assumption that antitoxin is present in its blood. Young specimens of the species, weighing about 500 grams, tolerate a single dose of the toxin sufficient to kill six thousand mice, whereas antitoxin does not appear in their blood until two months have elapsed.

The very great but not unlimited insusceptibility[13] of the fowl to tetanus affords one of the most striking examples of a natural immunity to the bacterial poison, that cannot be referred to the presence in its blood of an antitoxic or neutralizing agent. The amount of toxin required to kill a fowl weighing 2 kilograms is of the order that would kill nearly three million mice of average weight. Vaillard (1891) was the first to show that when toxin is injected into the peritoneal cavity of the fowl, almost all of it passes into the blood, where it is retained for a variable period. This investigator also proved that the tissues of this bird are able to produce considerable quantities of antitoxin.

It is of interest to know what becomes of the toxin that disappears from the blood stream of this animal. In regard to this point, Metchnikoff (p. 336) states that, "When fowls are killed at the stage when their blood becomes tetanogenic (as a result of the injection of the toxin into the peritoneal cavity), it can be demonstrated that their viscera are not capable of producing tetanus in susceptible animals except in so far as they contain blood. It is only the vascular organs, rich in blood, such as the spleen, liver, kidneys, thyroid gland, and bone marrow, that impart tetanus, and then only in so far as they have not been freed from blood. Of the various organs, only the genital glands, the ovaries or testes, absorb a certain amount of the injected toxin."[14] Metchnikoff's statements have been completely corroborated by Ransom (53) whose more extended researches were

[13] Courmont et Doyon: Compt. rend. Soc. biol., 1893, v, 841.

[14] See also Metchnikoff: Ann. l'Inst. Pasteur, 1897, xi, 801–809. It will be evident from our critical analysis of the work of Asakawa and Wolff-Eisner that we entertain serious doubts in respect to the correctness of Metchnikoff's statement that the genital glands actually absorb a "certain amount" of the injected toxin in a form that permits of its detection by means of bio-assays.

carried out without knowledge of Metchnikoff's prior work; the genital organs were not, however, tested by Ransom for retained toxin. This author remarks that the most favorable time for learning whether the toxin that has disappeared from the blood stream of fowls is stored ("Aufspeicherung") in their organs, is to examine them at a moment when the blood and lymph are quite or almost free from it. Numerous experiments were made in which fractions of a lethal dose, that induced no symptoms whatever of tetanus, were injected intravenously into fowls, and five days thereafter, at a time when the blood serum contained but little toxin, the following organs were examined for retained toxin: the lungs, kidneys, liver, spleen and heart, the brain and spinal cord, and the pectoral and leg muscles. These organs were ground up and emulsified with an equal weight of isotonic salt solution and allowed to stand for seventeen hours in the refrigerator, when the emulsions were centrifugated. The clear centrifugates were tested on mice for both toxin and antitoxin, in 0.5 cc. quantities. Not a trace of either of these two principles could be detected in *any of the emulsified organs*, with the exception, in one instance, of the lungs; but their small content of toxin was clearly due, as Ransom says, to retention of some of the slightly toxiferous blood. That the toxin did not escape from the body by way of the kidneys, in these experiments of Metchnikoff and Ransom, has been proved by Asakawa.

No careful studies with lethal and supralethal doses of toxin have been made in the case of the fowl to determine the point at which its organs cease to store it in a non-recoverable form. An experiment of ours in which a dose that killed a fowl in three days was injected intraperitoneally showed that fully 10 per cent of the toxin was present in the blood 48 hours after the injection. The experiment shows that the capacity of the fowl's tissues to store toxin is far from being unlimited, and lends support to our earlier statement, that once the tissues of an animal have bound the toxin to the extent of their capacity, the unbound excess is recoverable in its active form from the blood-lymph system. The manner in which the viscera and other organs of the fowl dispose of the toxin that has been withdrawn by them from the blood stream requires more extended study.[15]

[15] See the papers of Fermi and Pernossi and those of other authors cited by them, in which data are given in reference to the marked ability of the intestinal mucosa

The researches of two other authors, Asakawa and Wolff-Eisner (75), that bear on this question of the fixation of the toxin by the organized structures of the body, must be referred to here. The experiments of Asakawa, described in a paper that appeared practically at the same time as that of Metchnikoff, approached the subject in a manner that differs from the method employed by Metchnikoff and Ransom. This author injected two chickens subcutaneously with 210,000 mouse units of toxin, killed one of them 24 and the other 48 hours later, and then examined their emulsified organs for toxin, using mice as test animals. With the exception of the brain and spinal cord (although the spinal cord of one of the two fowls was found to contain a little toxin), all of the organs tested—the testicles, spleen, liver and muscles—contained very considerable quantities. This is not surprising, since the serum of the blood of both animals was highly toxic. However, in order to prove that his positive results could not be accounted for by retention of toxiferous blood by these organs, Asakawa injected a third chicken in the same manner, but in this case the vascular system was washed out by irrigating it with an 0.5 per cent sodium chloride solution. Unfortunately, the quantity of saline solution employed and the time involved in the irrigation were not stated.[16] Singularly enough, to quote Asakawa's words, "The results obtained in this third experiment were exactly like those of the two preceding ones." We are confident that all investigators who have had much experience with this highly virulent toxin will join us in refusing to accept Asakawa's conclusions, particularly in view of the results obtained by him in his third experiment. He believed that the organs of the fowl had fixed the toxin in a "recoverable form," because he succeeded in inducing tetanus by injecting emulsions of them into test animals. His positive results with organs,

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of the living guinea-pig and cat to render tetanus toxin innocuous. The fowl's intestine, according to these authors, is not endowed with this property. See also a recent article by R. Regamey: Ann. d. l'Inst. Pasteur, 1936, lvi, 87.

[16] The difficulty of removing all of the blood of an animal's body by irrigating its vascular mechanism with salt solution has been emphasized by a number of observers, some of whom found that after using as much as 30 to 40 liters of a saline solution, under constant pressure, at least 8 per cent of blood might still be retained. (For the literature, see Erlanger: Physiological Reviews, 1921, I, 177.)

like those obtained by Wolff-Eisner now to be described, are fully accounted for by the small amount of active and assayable toxin that still remained in their blood and lymph. When such organs are ground up and emulsified, the remanent toxin attaches itself to the finely divided particles of the disintegrated tissues and thereby offers a certain resistance to its removal, which can be overcome only by repeated washings. It is not surprising therefore that tetanus results when insufficiently washed emulsions are injected into test animals.

Wolff-Eisner carried out a most extensive series of researches, not only with chickens but with rabbits, guinea-pigs and frogs, in much the same manner as was done by Asakawa. The experiments on chickens will be found in Table B, page 222, of his paper, cited in the bibliography. The blood serum and the viscera of one fowl were tested three hours after intravenous injection of a known amount of toxin; in the case of the second fowl, two days after intravenous injection of the same amount; and in the third fowl, 8 days after subcutaneous injection. Wolff-Eisner sums up his findings in the case of the fowl by remarking (p. 217) that they were "analogous to those of Asakawa." His conclusions were vitiated by the identical errors to which attention has been called in connection with Asakawa's work.

It will be evident to the reader that both Asakawa and Wolff-Eisner join all of the other writers, whose work has been cited in the preceding sections of this paper, in believing that the biological test, or bio-assay method, when it gives positive readings, shows that toxin is fixed in an active and recoverable form by the tissues that have absorbed it.

VI. FINAL STATEMENT OF OUR VIEWS IN REGARD TO THE FIXATION OF THE TOXIN BY THE VARIOUS TISSUES OF THE BODY

From the time it was first shown that tetanus is a form of poisoning, caused by what has been designated tetanus toxin, it has been believed by investigators, without exception, that we have at our command methods of recovering the toxin in its native form from the various organs of the body that have been specifically affected by it as well as from those that are apparently less affected. The proof of this proposition seemed to be established beyond all controversy when it

was found that emulsions or extracts of the organs of tetanized animals (or of individual organs that had been injected with the toxin) reproduced, with exactness, all of the characteristic symptoms of the disease, when injected into the smaller highly susceptible animals, such as mice and guinea-pigs. The proof rests, therefore, on the outcome of a biological test—the basis of the bio-assay method described in Section II of our paper. When this test gave *positive* readings in experiments with the organs of tetanized animals, it was inferred that the toxin was actually *separable* from them in its active form and that it had acted in this form upon the organs specifically responsive to it, if not on others, such as the spleen and liver, extracts or emulsions of which were likewise capable of giving positive bio-assays when injected into test animals.

When the test gave *negative* readings, it was believed that the toxin was no longer detectable, because it had been rendered inert in some manner or that it had actually been destroyed in the course of its fixation. One apparent exception to this rule was seen in the fact that the brain and spinal cord gave no positive tests in the case of animals that had been tetanized with large doses of the toxin. And yet in these cases the central neurons, like the neuromuscular substance of the voluntary muscles, had undoubtedly taken up the poison in its active form, as was shown by the resulting tetanus.

The failure to induce tetanus in mice and guinea-pigs on inoculating them with emulsions of the spinal cord, the medulla or other parts of the neural axis taken from fatally tetanized rabbits, cats, or larger animals, was thought to be explainable on the assumption that the amount of toxin that had been taken up by these parts of the nervous system was too minute for its detection by even so delicate a test as that involved in the bio-assay method. Recourse was then had to the direct injection of relatively large amounts of toxin into the cervical or lumbar part of the spinal cord of cats or rabbits; after 48 hours or less, when symptoms of tetanus were well marked, the animals were killed, the spinal cord was removed and suitably treated emulsions or extracts of it were injected into mice, which now responded with either a local or a fatal tetanus. At the time these tests were made, almost the entire amount of the original toxin had been removed by the blood stream from the injected area, so that, accord-

ing to our calculations, only a small part of 1 per cent remained behind (see Fröhlich and Meyer, pp. 60 and 61). It was this small residue, so easily demonstrable by its action on mice, that was wrongly supposed to have induced the symptoms of tetanus, which were really due to the fixed and non-demonstrable fraction of the toxin.

Such experiments, in which fatal amounts of toxin were injected directly into the spinal cord, cannot be brought into agreement with the much larger number of experiments cited in the literature, in which as many as 100 fatal doses of toxin were injected intravenously, and where, in spite of this great excess, *no positive biological test could be obtained with pieces of the spinal cord or brain.*

Experiments of the kind just described, in which considerable quantities of toxin were injected directly into the spinal cord, and in which positive bio-assays were obtained, are subject to criticism. If more than 99 per cent of the injected toxin is carried away from the spinal cord by the blood stream in the course of 48 hours, why could not the rest of it (with the exception of what had been irretrievably fixed) have been removed in the same manner had more time been given? It is well known that when a considerable amount of toxin is injected at a single point, underneath the skin, into the neighborhood of tendons, or into the muscles of an extremity, it requires three or more days for the removal of every detectable trace of it from the injected site. *As in all other instances, so in the case of the injected spinal cord, it is only the fixed, non-recoverable and non-assayable toxin that acts upon the cells of an organ.*

The most conclusive argument, however, against the validity of the experiments with the spinal cord, cited above, is found in the incontrovertible fact that but a short time is required for the enduring fixation of the toxin by the cells that have accepted it. In the experiments under consideration, the cells of the injected areas of the spinal cord had already permanently fixed their quotum of the toxin, forty hours or thereabouts before the illusory and misinterpreted bio-assays were made.

In passing we may point out that the reason why the many investigators, who have never failed to secure evidence of the presence of active toxin in many other organs of the body, have never found a trace of it in parts of the brain or spinal cord, is as follows: The numerous large arteries that supply the central nervous system and the many

sinuses and veins that carry away the blood from this part of the body are located outside the substance of the brain and spinal cord. When these organs are removed soon after death they are found to be pale and relatively bloodless, as compared with the spleen or liver, for example. How strikingly different is a cross section of the lumbar cord, when separated from its meningeal covering, from a cross section of the spleen or liver when examined at the same time after death! Were the arrangement of arteries, veins and the venous sinuses the same in the substance of the brain and spinal cord as in the organs just named, and were they therefore equally well filled with blood when removed from the body, the many investigators who have had no difficulty in finding separable and assayable toxin in the spleen and liver, would have had no greater difficulty in finding it in the brain stem or in the spinal cord.

So too the muscles of the animals employed in laboratory experiments, such as those of the dog, the rabbit, and others, are also comparatively bloodless organs at the time of death, and this again accounts for the frequent notations in the protocols of so many investigators that the voluntary muscles contained only a small amount of the toxin in an active form.

A positive biological test, as we have seen, has been interpreted unanimously, though mistakenly, as furnishing proof for the contention set forth above. Investigators have also been unanimous in believing that a negative biological test is of equal significance, but from an opposite point of view. Numerous instances have been given in preceding sections, in which negative biological tests were believed to furnish incontrovertible evidence in support of the conclusion that the assayed organs had either destroyed their quotas of active toxin, or that they had so altered it as to render it innocuous, not only for themselves, but also for test animals into which they were injected. Both Metchnikoff and Ransom held this opinion in respect to the negative results that were obtained by them in their experiments on fowls cited above, but both of these authors, as is clearly evident from their other papers, also agreed with their contemporaries in believing that a positive biological test enables an investigator to prove that the toxin is present in many organs in an active, and at the same time, an assayable form.

The experimental evidence at hand supports the belief that the

toxin is always fixed in a non-recoverable and non-assayable form by all of the organs of the body, that have an affinity for it, irrespective of their functional rôle or of the manner in which it influences them. In other words, no organ that has once fixed the toxin can give a positive biological test for it. It is easily shown that this fixation begins to take place very soon after the injection of a sublethal, or of a fatal dose of the toxin, as well as in those instances where the blood and lymph contain it in large excess after many lethal doses have been injected. This incontrovertible fact alone suffices to discredit all of the deductions that have been based on positive biological tests obtained with organs subsequent to the fixation of toxin by their cells. The bio-assay method is of the greatest value in enabling us to show how much of an active toxin was offered to an individual organ or to the body as a whole; the fact that toxin is actually present in organs, in an active, though non-removable form, must be proved by an eventually occurring tetanus.

Further experiments to prove that the toxin is soon so firmly and irretrievably fixed by the susceptible and other tissues of the body that it can no longer be removed from a living animal by repeatedly infusing it with normal blood. There is another method of proving the fixation of toxin in a non-recoverable and non-assayable form—a method in which the amount of it that has thus been fixed is estimated in terms of the number of units that have escaped from the blood stream, loss by excretion being regarded as negligible. To this end quantitative bio-assays of the amount of toxin in the blood at the beginning of the experiment and at stated intervals throughout its entire course are made; it will become evident as we proceed that it would be superfluous to make biological tests with the organs that have irretrievably fixed the toxin, in view of the evidence that has been adduced that when they are free from toxiferous blood and lymph positive biological tests are no longer obtainable.

A number of experiments of this kind have been made by us, the details of which will be described in a later paper.[17] The original object of our experiments was to determine how long a time must elapse

[17] In making these experiments, which had to be carried out aseptically, we have been favored with the cooperation of Dr. August F. Jonas, Jr., of the Surgical Department of the Johns Hopkins University.

after the intravenous injection of from 1½ to 3 lethal doses of toxin into dogs, before their organs absorb and fix enough of it so that death will inevitably follow. The experiments were made with small dogs of approximately the same weight, all of which had recently attained maturity. The amounts of highly active filtrates that were injected had been well assayed in terms of guinea-pig units. The exact time of the intravenous injection was noted and after a certain time had elapsed, the animals were lightly etherized and subjected to the following procedures. The femoral artery and the femoral vein were cannulated. Suitable arrangements were then made for repeatedly bleeding the animals by the femoral artery to the danger point and for immediately infusing them with amounts of heparinized normal dog's blood, equal to those that had been withdrawn from the artery. The amount of normal dog's blood at hand for repeated infusions was 2½ or more liters.

Let us take, for example, an experiment in which 9,702 LD50 guinea-pig units of toxin were injected intravenously into a dog weighing 6.9 kilograms. Exactly 4 hours and 41 minutes after the intravenous injection of this number of units of the toxin, approximately equivalent to 3 lethal doses, the arterial cannula was opened and 340 cc. of the blood were allowed to flow out. Immediately thereupon, the animal was infused by way of the femoral vein with an equal volume of normal heparinized blood, under a pressure of about 3 feet of blood, with an inflow period of 3 minutes. After allowing an interval of 6 to 10 minutes for the proper distribution of the infused blood the arterial cannula was again opened and approximately the same amount of blood as before allowed to escape from the artery. Immediately, as before, a volume of the normal heparinized blood equal to that removed by the artery was introduced by the femoral vein. The operations of bleeding and infusion of normal blood, with duly recorded brief periods of rest, were repeated seven times in all. The animal was lightly etherized during the entire operation and recovered so completely and was so little inconvenienced by the repeated bleedings and infusions that we confidently believe these could have been repeated many more times. The dog passed through a five-day period of incubation without any sign of disturbed health, and showed the first beginnings of descending tetanus at the end of

the 5th day. The symptoms of generalized tetanus increased in severity on the following days and became so severe by the 8th day that we believed the animal could not recover. It was certainly close to death, with muscular rigidity, inability to walk, pronounced trismus, profuse salivation, and difficulty in swallowing water or finely chopped meat. These severe symptoms continued for two days (from the 8th to the 10th day), after which the animal began slowly to improve; it was "gassed" a month later, when it still showed marked signs of the emaciation always seen in severe cases of experimental tetanus. A control animal of the same weight, that had received an equal amount of the toxin per kilogram of body weight, manifested the first symptoms of tetanus after an incubation period of three days, and succumbed to the disease on the 8th day.

The blood withdrawn at each of the seven bleedings was kept in a separate flask and the amount of toxin contained in each was assayed in terms of guinea-pig units. The quantity of toxin removed from the animal's blood in the first bleeding (340 cc.) amounted to 4,218 guinea-pig units, indicating that 51 per cent of the toxin present at the time in the circulation had been removed at this withdrawal of the blood; the amount of toxin here withdrawn represents 43 per cent of that originally injected. Ten minutes after the last, or seventh infusion of normal dog's blood, 30 cc. of the animal's blood was withdrawn and its content of toxin determined. On the assumption that the blood of a dog constitutes 10 per cent of its body weight, our bioassays show that the total amount of toxin that remained in the blood stream after the seventh bleeding and the seventh infusion of normal blood amounted to only 158 guinea-pig units. When this small residue is compared with the 9,702 units originally injected, it will be seen that it alone would be entirely ineffective in inducing any symptoms of tetanus in a dog of the given weight, as we know from many previous experiments. Now, it is interesting to note that in the course of the seven bleedings, which required one hour and 22 minutes, we actually removed 6,444 guinea-pig units of toxin. If to this amount we add the 158 units of toxin left in the animal's blood after the 7th bleeding, we learn that we can account, at the end of the experiment, for 6,602 of the 9,702 guinea-pig units originally injected. On the day following the operation the toxin content of the dog's

blood was practically at the same level as after the seventh and last infusion; from this time on this small amount of remanent toxin gradually disappeared until only a trace of it was detectable on the sixth day.

A simple calculation shows that in the entire course of our experiment, the dog's organized tissues had extracted from the blood, and fixed in a non-recoverable manner, about 3,100 of the 9,702 units of toxin previously injected. There are indications that the relatively insusceptible animals used in our experiments continued to fix some of the toxin during the time that was taken up in the seven bleedings and infusions, particularly during the early period when the blood still contained relatively much toxin. Even the 158 units (23 per kg.) left in the blood at the close of the experiment were finally absorbed and fixed by the tissues. This last fraction, however, could undoubtedly have been removed by another three bleedings and infusions, to the point at which bio-assays fail.

The injected 9,702 units of toxin represented three lethal doses. The 3,100 units that were fixed by the tissues, in the time stated and in a manner that resisted our attempts to remove it from them by repeated infusions of normal blood, amounted, therefore, to a little less than a lethal dose for the dog in question. It is of interest to note, that none of this fixed and all but fatally acting fraction of the injected total found its way back again into the blood stream at the end of the experiment, when the blood was practically free from toxin; this need not, however, occasion surprise, in view of the many instances given in our paper of so firm a fixation of toxin by the organs of tetanized animals that it could no longer be released by the blood and tissues of normal mice and guinea-pigs.

In a previous experiment, in which 1 hour and 30 minutes had been allowed to elapse between the intravenous injection of the toxin and the first withdrawal of arterial blood, and in which also the procedure above outlined was repeated seven times, only light symptoms of tetanus that persisted for four or five days resulted.

In a third experiment of the same kind as the one just described, that came so near to ending fatally, a 10-kilogram dog received nearly 9,000 guinea-pig units intravenously, as before, and was allowed to run about for 7 hours. It was then lightly anesthetized with ether,

the femoral artery and vein were cannulated and the apparatus required for the repeated bleedings and infusions of normal blood assembled, all of which procedures consumed an additional 41 minutes prior to the first withdrawal of arterial blood. In this animal the amount of toxin that was still retained after the final infusion of blood was even less per kilogram of body weight than was the case with the animal in the 4 hour and 41 minute experiment; the retained toxin here amounted to 17 guinea-pig units per kilogram as compared with 23 units per kilogram in the earlier experiment. The total amount of toxin that was here so firmly bound, in 7 hours and 41 minutes, as to withstand repeated infusions with normal dog's blood, was nearly double that bound by the former animal, being equivalent to 69 per cent of the injected amount, or close to $1\frac{1}{3}$ lethal doses. This animal died at approximately the same time, on the 7th day, as the control animal that had received an equal dose of the toxin.

The experiments we have so far made in this connection lead us to believe that after the injection intravenously, at one time, of only $1\frac{1}{2}$ to 3 lethal doses of toxin into dogs, there is required a period of about five hours for the fixation in an irreversible manner of a sufficient amount of the toxin to ensure death. It will be of interest to learn, as we continue our researches, to what extent the time required for the fixation of a fatal quantity is shortened consequent to the injection of such excessive amounts as lead to death in a few days—amounts that frequently come into play in the natural tetanus of domestic animals and of man.

Our results are in agreement with the earlier observations of Dönitz and others, who established the fact that after the intravenous injection at one time of one or more fatal doses of toxin into rabbits, the subsequent injection of even excessive amounts of antitoxin is powerless to prevent death, in case five hours or more are allowed to intervene between the two injections.

The experiments on dogs here described, taken with those in which it was proved that the toxin is not conveyed to the central nervous system by peripheral nerves (Paper III), discredit, it appears to us, the widely accepted theory that there exists a blood-brain barrier that prevents the toxin from gaining access to the susceptible neurons of the neural axis. On the contrary, our experiments prove that the

responsive parts of the central nervous system begin to absorb and irretrievably fix their own small quotum of this slowly diffusible protein-like poison, from the moment it reaches them via the blood stream, exactly as do the other organs of the body that have an affinity for it.

Equally unsupported is the still prevailing and accepted belief that the toxin acts upon the central neurons immediately it has reached them via the motor axons of peripheral nerves, and that the time involved in this conveyance from the terminals of the motor axons to the central neurons accounts for the delayed central action of the toxin—the so-called "period of incubation." It can easily be shown, however, that a long period of latency, or incubation, intervenes, both in the case of the neuromuscular substance of the voluntary muscles and the central neurons, after direct injection of very small amounts of toxin into them,[18] and that this delayed response is the result of events occurring in these structures themselves and operative there from the very moment when the toxin reaches a certain concentration in them. All of these points will come up again in later papers.

Nearly forty years ago, Decroly and Ronsse attempted to show that the tissues of the rabbit (an animal that is about twice as insusceptible to the toxin as the dog) were able to bind a fatal amount of the toxin in $2\frac{1}{2}$ minutes, or less, after the intravenous injection of a lethal dose. Under the same conditions, a fatal quantity of diphtheria toxin was thought to be bound in $7\frac{1}{2}$ minutes and of cobra venom in 9 minutes. While we have not repeated the experiments of these authors, we are by no means convinced that the organs of rabbits can fix a lethal quantity of tetanus toxin in the time stated, particularly when the amount injected was believed to have constituted only a single lethal dose, as was shown by the amount needed to kill control animals. We cannot here consider in detail the experiments of these observers, which were carried on in a manner that differed entirely from that employed by us; but we may be permitted to point out that no attempt was made by them to determine the amount of the injected toxin in terms of guinea-pig or mouse units. Neither was there any

[18] See Paper III for latent period in the case of local tetanus, and a forthcoming paper on direct injections of toxin into the spinal cord.

attempt made to assay the amount of toxin removed in the course of a single sharp bleeding and in the course of two subsequent injections and withdrawals of a saline solution equal in volume to the amount of blood withdrawn in the single sharp bleeding; after the second withdrawal of the saline solution an equivalent amount of normal rabbit's blood was injected. In addition to this major defect in the work of Decroly and Ronsse, a study of their protocols, especially with reference to the controls, also brings to light a number of inconsistencies that lead to the suspicion that the tissues of the rabbit, which are endowed with the ability to store 10 or 12 lethal doses of the toxin in the course of 20 hours, are not able to fix a lethal amount of the toxin in $2\frac{1}{2}$ minutes or less, when only one "lethal dose" is injected.

VII. SUMMARY

We have prepared the following summary of the conclusions that we have drawn from our own experiments and those of others, for the convenience of readers who are less interested in experimental details than in the conclusions that are derived from them.

1. In the introduction a brief account is given of the opinions held, in the pre-bacterial era, by leading physicians and surgeons in regard to the true nature of tetanus. The rise and dominance of certain errors that led to a false pathogeny of tetanus are also there commented upon.

2. In Section II we have described in some detail our method of assaying the toxin present in culture filtrates, blood and lymph, and the manner in which statistical analysis was applied to the numerical data obtained in numerous bio-assays. We closed the section with the protocols of an experiment on a sheep, by way of illustrating how our quantitative data were gathered, analyzed and interpreted in a particular instance.

3. Many experimental data have been given above, proving that free toxin cannot be shown to be present in the blood of animals so highly susceptible to it as the horse, the sheep and the guinea-pig, when these have been inoculated, subcutaneously or intramuscularly, with *a single dose that just suffices to kill them*, (without setting up the requirement of a definite time limit for the fatal outcome).

4. *Rapid transference of an excess of toxin to the blood-lymph system.*

If we consider first our experiments on sheep and on the horse (Table III) we find that one result of injecting, subcutaneously or intramuscularly, somewhat more than the lowest single fatal dose, is the rapid transference of the entire surplus of the injected toxin above this lethal dose, or, at least the greater part of it, to the blood and lymph. As more and more of the toxin is injected, practically the entire excess, over and above one lethal dose, continues to accumulate in these two fluids, to which must be added the small non-determinable, and perhaps entirely negligible amount present in the very narrow tissue spaces of the body. It is apparent from our protocols and tables that when several lethal doses have been injected at one time, by one route or another, into sheep, the blood and lymph together (once equality as regards their toxin content has been attained) actually harbor much more than 90 per cent of the injected toxin, and continue to do so for a number of days until death occurs. In other words, when a supralethal amount of toxin, equivalent to 10 minimal lethal doses, let us say, is injected into one of these animals, all but about one lethal dose, or a little more, is recoverable from the blood-lymph system which serves as a reservoir for the other unabsorbed and unchanged 9 lethal doses. But while there is no experimental evidence for any measurably greater absorption of toxin by the specifically affected, or even the other organs of the sheep, at such higher levels of concentration in the blood and lymph, we nevertheless have proof that *some* additional absorption must have occurred. The proof is found in the fact that four, five or ten lethal doses, for example, in place of only one, are somewhat more quickly fatal than a single lethal dose. No direct linear connection, however, can be established between the increase of the dosage and the times at which death occurs.

Many experimenters have succeeded in detecting the toxin in the blood of people with a fatal tetanus, by means of bio-assays with mice and guinea-pigs, while many others have failed entirely to do so in instances of the disease that also terminated fatally. It is evident, then, that we encounter the same conditions in human beings with tetanus as in certain of the more susceptible animals, namely, that the blood may contain no demonstrable amount of toxin when just enough of it to insure death has entered the system, by the subcutaneous or intramuscular route, but that *very much of it will be present when a considerable number of lethal doses are operative.*

5. *Secondary actions of the toxin.* When the death of an animal occurs soon after intravenous injection of a thousand or more lethal doses (within nine or ten hours, as in the case of the guinea-pig), it may justifiably be assumed that secondary actions of the toxin on other organs of the body than the voluntary muscles and the central neurons, play a rôle in hastening the fatal outcome. Not all animals, however, die as quickly as does the guinea-pig after the injection of massive doses of the toxin, as is shown by the experiment of Marie and Morax, who injected one thousand or more lethal doses of precipitated toxin into the gastrocnemius muscle of a pony and found that it survived for almost two days.

We have reason to believe that some at least of the viscera, notably the liver, are profoundly affected by their retained and non-detectable toxin, more especially when very large doses of it come into play in acute cases, or when chronic poisoning results from the repeated administration of sublethal doses. The literature dealing with the production of antitoxic serum cites instances of horses that died while undergoing immunization, whose liver, at autopsy, was found to be ruptured. Another reason for believing that the toxin can induce profound metabolic alterations in the body is found in the well established fact, repeatedly verified by us, that experimental animals lose weight in the course of a severe and rather prolonged but non-fatal tetanus, at a time when food is still avidly consumed with undiminished appetite. This is certainly the case with the dog. Then, too, physicians of the widest experience have often commented on the asthenia and the appalling emaciation that may befall the most robust of men during a prolonged tetanus; the shocking condition of such patients is not thought to be fully accounted for by the lessened intake of food and water. We conclude, then, that when the toxin reaches a certain concentration in those of the so-called non-reactive organs whose cells are capable of absorbing it, some of them may be deranged by it in a manner consonant with their special functions, just as the voluntary muscles and the neurons of the spinal cord, medulla and midbrain respond to it in their own characteristic manner.

6. *Time of death predetermined.* A point of great interest implicit in our blood assays, but which is brought to light only when these are viewed in connection with other experiments, is the following: Whether a moderate amount of tetanus toxin, represented by a single

lethal dose, or, whether a very considerable excess of it, as after several lethal doses, is present in the blood-lymph system, the actual time of death is definitely determined in each case by the absolute amount that has been irretrievably fixed by the various organs of the body in the first few hours after the requisite threshold concentration in the tissue spaces has been attained or surpassed. This point is proved by the quantitative experiments with dogs described in Section VI. The events of these hours initiate the period of incubation, determine its duration, and are decisive for life or death. This law as here stated is one that applies to many poisons, but it is of especial importance in animal experiments with the bacterial, the higher plant and animal poisons and the numerous viruses, whose action is characterized, like that of tetanus toxin, by a more or less prolonged period of incubation, and which, in due course, initiates and determines the production of antibodies of a specific nature; like tetanus toxin, many of these poisons also circulate in the blood stream for a longer or shorter time and are transferred from it and fixed upon the specifically reactive and the other organs of the body.

7. *There is no blood-brain barrier for the toxin.* The quantitative blood assays made in the course of the experiments with dogs described in Section VI, taken with the experiments in which it was proved that the toxin is not conveyed to the central nervous system by peripheral nerves (Paper III), discredit, it appears to us, the widely accepted theory that there exists a blood-brain barrier that prevents the toxin from gaining access to the susceptible neurons of the neural axis. On the contrary, our experiments prove that the responsive parts of the central nervous system begin to absorb and irretrievably fix their own quotum of this slowly diffusible protein-like poison, from the very moment it reaches them via the blood stream, exactly as do the other organs of the body that have an affinity for it. The full details of the experimental proof for the latter statement will be given in a later paper.

8. *The varying ability of the different animal species to dispose of the toxin.* The fact that the excess of toxin above that required to kill finds its way, when injected under the skin or into the muscles, almost *in toto into the blood and lymph of the highly susceptible animals*, suggests at once that the various organs have now largely exhausted

the power to dispose of this excess (in a manner that evades detection), in contrast to their power to dispose of all of the toxin of a single lethal dose. It has been shown that after the subcutaneous or intramuscular injection of a lethal dose or less into any one of a number of the very highly, or into some, at least, of the less susceptible animals, none of the toxin can be detected in their blood or in any of their organs by our present methods. It has been repeatedly demonstrated in the past that this failure to detect the toxin cannot be accounted for on the assumption that it is largely eliminated by the excretory organs (this point has been considered in the body of our paper); nor is this failure to be explained by the disappearance of the toxin in the course of a rapid antibody production.

It is known, moreover, that, in a number of animals, such as some of the cold-blooded animals and the fowl, so refractory to tetanus that very large amounts of its toxin are required to kill them, the blood-lymph system accumulates and harbors for a considerable time even the smallest fraction of an injected lethal dose. It is not our purpose, however, to consider at this time the toxin-retaining power of the blood-lymph reservoir of these very refractory animals for varying doses of it, but to confine our remarks more particularly to the more susceptible animals with which we have experimented. Its continued retention in their case by this reservoir (elimination by the secretory organs being excluded) cannot be explained on the assumption that the accumulated toxin *no longer has access to those organs and tissues that were originally so operative in fixing and hence in concealing from our bio-assays the entire amount of a single lethal dose.* When the blood is laden with an excessive amount of toxin that circulates in it for many days, it must traverse the narrow tissue spaces, that serve as its diffusion pathway from the blood capillaries to those of the lymphatic system. It therefore always lies, so to speak, on the doorstep of the cells of those organs, whose ability to store a single lethal dose, in a non-assayable form, must be conceded; if these cells could have continued to absorb and dispose of many additional lethal doses, the toxin could not have accumulated in the blood of sheep, horses, and other animals in the amounts found by us in our experiments.

All of our experiments support the conclusion that, when the blood

and lymph of sheep or horses or of other very susceptible animals retain excessive quantities of the toxin beyond a lethal dose throughout many days, this retention is not due to a temporary impermeability of the capillaries for the toxin, but rather to the fact that the cells of the various organs of the body are, for the time being at least, no longer capable of taking up additional amounts of it, although it is freely offered to them in the tissue spaces. Our own experiments as well as those of our predecessors prove that these spaces present no obstacle to the free passage of the toxin from the blood to the lymphatic capillaries.

The varying ability of different species in respect to the time required for the disposal of supralethal quantities of the toxin, short of hundreds of lethal doses, is well illustrated in comparative experiments with the sheep and the rabbit. It is found that when the two animals are inoculated, in one way or another, with, let us say, ten lethal doses, the blood and lymph of the sheep continue to retain most of the toxin until death occurs, while those of the rabbit have already lost practically all of it in about twenty hours; that is to say, as early as the first part of the incubation period. This strikingly contrasting retaining power of the blood and lymph of the sheep and the rabbit, subsequent to the injection of supralethal doses of the toxin, is not confined, as our records show, to this particular pair of animals. What an enormous capacity the blood-lymph system of the horse has for taking up and retaining hundreds of lethal doses of the toxin, in excess of the single fatal dose, is shown in the case of the St. Louis horse with natural tetanus cited in the text.

9. *Significance of toxin in the blood.* Once the receptor substance of the voluntary muscles and the receptive cells of the central and autonomic nervous systems, as well as those of the so-called non-reactive organs of the body, have been given the opportunity and the requisite short time to absorb and fix the relative quotas of toxin that suffice, or more than suffice, to insure death, the excess of toxin that may happen to be present in the blood-lymph system is no longer of material significance *quoad vitam*. The amount of the toxin that is fixed upon the highly responsive organs constitutes but a minute fraction of the entire lethal dose, the vastly larger part of which is coincidently fixed by the less responsive organs. We have

already alluded to the possibility that when enormous doses of the toxin are injected, particularly into animals whose tissues have the ability to fix large amounts of it, the time of death may be hastened more by events due to the fixation of such excessive amounts by the non-responsive organs of the body, such as the viscera, than by any excess that has been taken up by the specifically responsive organs.

10. *What becomes of the toxin that is taken up by the cells of the various organs?* We have called attention to our conviction that the toxin cannot be detected in, or recovered from, any of the organs of the body after the toxiferous blood and lymph have been completely removed from them. The results of our analyses have convinced us that the blood-lymph reservoir is the only part of the body in which the toxin is present in its unimpaired and natural state. Withdrawn from this reservoir, it is found to be as lethal for test animals and as readily assayable as a sample of a control toxin that has been kept in the refrigerator. As long, then, as the toxin circulates in the blood and lymph it is recoverable from these fluids and from *incompletely washed tissues* and is identifiable as active toxin. Not so, however, when it has been taken up either by the specifically reacting, or the other organs, of the body; both classes of organs bind it in a manner that evades detection by the bio-assay method. Experimental evidence at hand, as was pointed out in Section VI, supports the belief that the toxin is always fixed in a non-recoverable and non-assayable form and in a relatively short time, by all of the organs of the body that have an affinity for it, irrespective of their functional rôle or of the manner in which it influences them. This fixation begins to take place the moment the toxin comes into contact with cells of the various organs. *An organ that has once fixed the toxin can give a positive biological test for it only so long as it contains toxiferous blood and lymph.*

Our statement as to the practically concurrent fixation of a fatal dose of *blood-borne toxin*, not only by the neurons of the central nervous system and the neuro-muscular terminals of the more susceptible voluntary muscles, but also by the other organs of the body that have a chemical affinity for it, is not accepted by either the many adherents of the nerve transport theory or by its few critics, all of whom hold that the "non-specifically affected organs of the body"

can saturate themselves with this blood-borne "neurotropic poison," the while the responsive neurons of the central nervous system must await its tardier arrival via a motor nerve, as for example, the long sciatic nerve of a horse.

Wolff-Eisner (1908), who reviewed the opinions of Ehrlich, Roux and Borrel and others in reference to what these authors called "the deviation" of the toxin from the vital centers to less important organs, based his theory of the underlying cause of the period of incubation on an elaboration of these earlier ideas. His experiments led him to believe that incubation is a function of "the attracting and binding power" of the non-reactive organs of the body; as long as these organs can continue to "attract and bind" the toxin, so long lasts the period of incubation. Only when these retaining organs have been fully saturated with the blood-borne toxin, can the central neurons receive enough of it to affect them; thereupon, the symptoms of tetanus, a purely central disease, according to our author, appear without further delay. We believe it to be unnecessary to comment on the untenability of his theories.

If the toxin that has been fixed by the organs of either a highly susceptible or one of the less susceptible animals, should happen to undergo one or more alterations of its molecular structure, in the course of its action on their cells, we might find ourselves unable to recognize alteration-products, even if we had them in hand; in a word, we may be dealing here with a toxic substance that loses its chemical identity, so to speak, in the act of exerting its deleterious influence upon the body. Were an action of this kind to take place, the process involved should finally reach a limit, as it does in the case of animals such as the fowl, that tolerate enormous doses of the toxin. We surmise that the mystery in respect to the fate of the fixed toxin may possibly be solved by means of well planned experiments on the fowl and other animals of its class.

It is well to bear in mind that our toxin, which may well turn out to be a polypeptide, must be classed with those poisons that *ordinarily* induce no definitely recognizable pathological tissue changes. A very large number of human beings and animals with tetanus are finally restored to health without residual damage to any organ; and it seems to be well established that the functional changes and such

cellular alterations, if any, that may have appeared in the course of a non-fatal tetanus, are of a reversible character. There can be no doubt, then, that the very cells of the body that are so specifically and dangerously affected by the toxin (to speak here only of this type of cells) have the power to fix and yet finally to destroy or to render innocuous, in some unknown manner, sublethal doses of it. This is but another example of a general property of protoplasm—one that is operative to some extent in every case of tetanus and in varying degree in the different animal species.

The ability of animals to fix the toxin in a non-recoverable manner varies within the widest limits, as is evident on comparing the very small amount of toxin required to kill a horse or one of the other highly susceptible species of animals, with the truly enormous quantity that constitutes a fatal dose for the fowl, the alligator or other members of their class. It is not permissible, therefore, to assume that the results obtained in experiments with the animals of a particular species will hold for those of another species, even when the two differ but little in their susceptibility to the toxin, as in the case of the dog and the rabbit. In concluding this summary we may again say that tetanus toxin is recoverable, in its native form, from the blood and lymph only; *the part of it that has been truly fixed by the cellular or other elements of the body, inclusive of those upon which it acts in a highly specific manner, is not recoverable, either as such or in the form of a derivative, by methods now at our disposal.* The latter part of this broad statement runs contrary to the accepted belief. It is the true nature of the fixation of toxin by the various tissues of the body that now baffles explanation. Here is encountered the most perplexing and perhaps the most important problem of tetanus. Its solution must be attempted, if for no other reason than its bearing on the contingent and inseparable problem of antitoxin formation.

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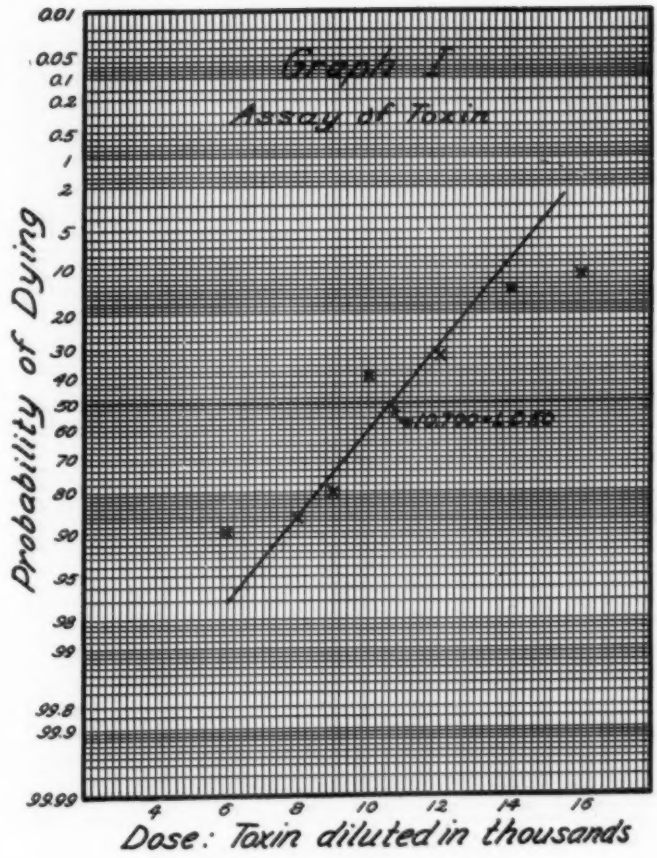
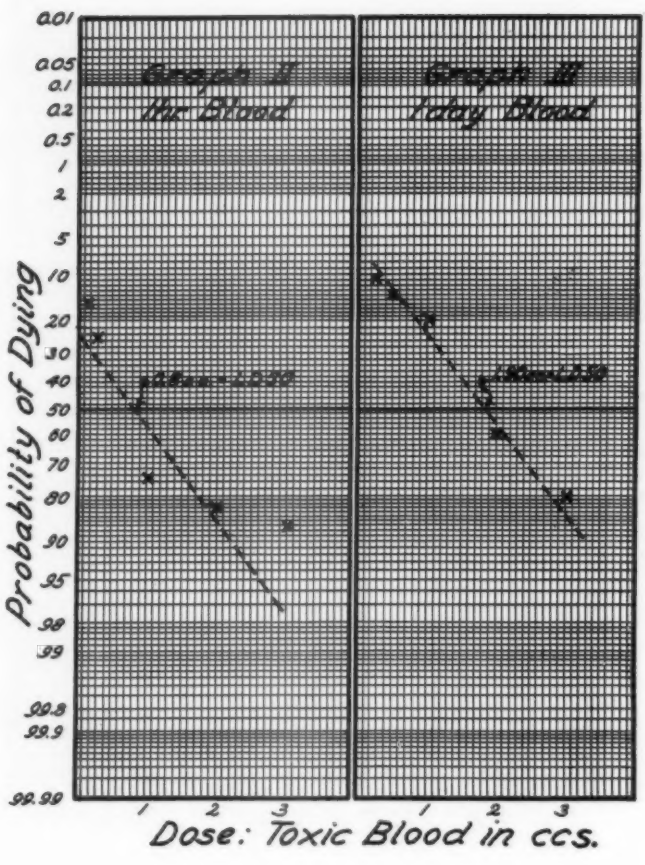
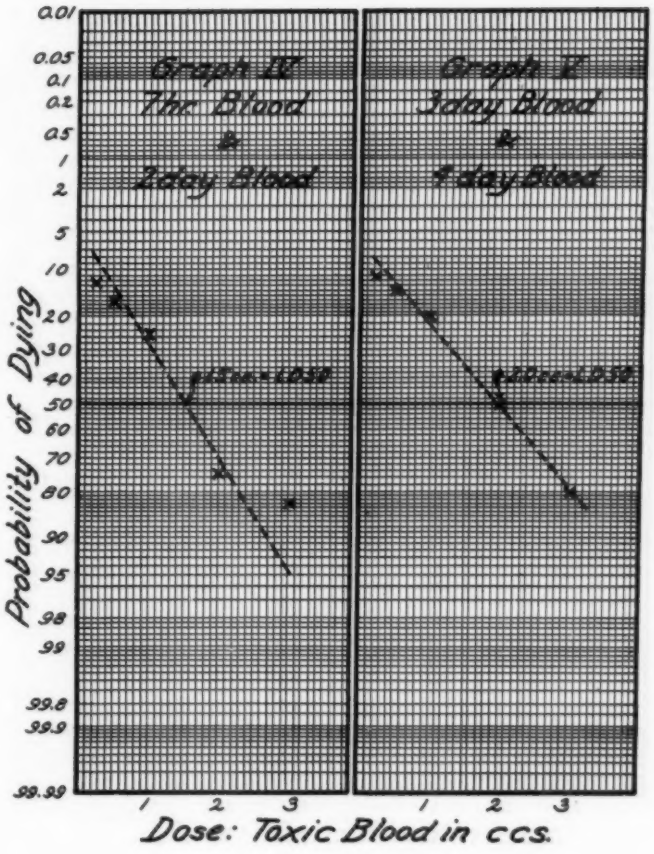

Graph 1
Assay of Toxin
Probability of Dying
0.01
0.05
0.1
0.2
0.5
1
2
5
10
20
30
40
50
60
70
80
90
95
98
99
99.8
99.9
99.99
4
6
8
10
12
14
16
Dose: Toxin diluted in thousands


Probability of Dying
0.01
0.05
0.1
0.2
0.5
1
2
5
10
20
30
40
50
60
70
80
90
95
98
99
99.8
99.9
99.99
Dose: Toxic Blood in ccs.


Graph IV.
7 hr. Blood
&
2 day Blood
Graph V.
3 day Blood
&
4 day Blood
Probability of Dying
0.01
0.05
0.1
0.2
0.5
1
2
5
10
20
30
40
50
60
70
80
90
95
98
99
99.8
99.9
99.99
1
2
3
Dose: Toxic Blood in ccs.